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DIROFILARIA IMMITIS AND *ANGIOSTRONGYLUS VASORUM*: EPIDEMIOLOGY AND IMPACT OF MAJOR HEARTWORMS IN CARNIVORES IN PORTUGAL

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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Sanidade Animal

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2017

"*I have been impressed with the urgency of doing.*
Knowing is not enough; we must apply.
Being willing is not enough; we must do."

Leonardo Da Vinci

This work is dedicated to my family,
for all their support, optimism and understanding.

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European Network for Neglected Vectors and Vector-Borne Infections

Title: *Dirofilaria immitis* and *Angiostrongylus vasorum*: epidemiology and impact of major heartworms in carnivores in Portugal

ABSTRACT

Cardiopulmonary nematodes, *Dirofilaria immitis* and *Angiostrongylus vasorum*, are severe and life-threatening parasites that are increasingly reported throughout Europe. However, in Portugal, accurate data on both illnesses is scarce, hampering the awareness and the implementation of effective prevention and control strategies. A multidisciplinary study was therefore designed to characterize and assess the current situation regarding dirofilariosis and angiostrongylosis in carnivores from Portugal.

A national survey was conducted to assess the prevalence and distribution of *D. immitis* and *A. vasorum* in canine and red fox populations in Portugal, confirming the occurrence of both diseases in canids either from northern, central and southern regions. An overall prevalence of 11.9% and 0.7% dogs, and 8.5% and 12.7% foxes were positive to *D. immitis* and *A. vasorum*, respectively. Additionally, a high prevalence of *D. immitis* was found in pinnipeds kept at an oceanographic park in the Algarve region where the South African fur seal was also reported as a new host for *D. immitis* infection. DNA of the endosymbiont bacterium *Wolbachia pipientis* was detected in dogs naturally infected with *D. immitis* in Portugal. The transmission risk period for *Dirofilaria* spp. in Portugal was estimated, showing that although transmission is markedly seasonal, the necessary climatic factors are starting earlier and lasting longer than the summer. A new minimally invasive surgical technique was developed to extract *D. immitis* adult worms from the hearts of dogs through transjugular catheterization with a non-traumatic snare. A questionnaire conducted on Portuguese pet owners showed that the majority deworm their dogs at irregular and consequently ineffective intervals and their level of knowledge about parasites and parasitic diseases is low.

Although exposure may differ depending on the region of Portugal, the likelihood of cardiopulmonary dirofilariosis and angiostrongylosis is considerable nationwide. Active surveillance, increasing awareness and regular preventive measures are crucial to control cardiopulmonary parasitic diseases in carnivores in the country.

Keywords: *Angiostrongylus vasorum, Dirofilaria immitis*, epidemiology, Portugal, carnivores.

Título da Tese: *Dirofilaria immitis* e *Angiostrongylus vasorum*: epidemiologia e impacto dos principais nemátodes cardiopulmonares em carnívoros em Portugal

RESUMO

A dirofilariose e a angiostrongilose são doenças parasitárias crescentemente notificadas em toda a Europa, representando uma ameaça grave para a saúde animal. São causadas respetivamente pelos nemátodes cardiopulmonares *Dirofilaria immitis* (agente transmitido por mosquitos culicídeos) e *Angiostrongylus vasorum* (agente transmitido por moluscos gastrópodes). Os fatores subjacentes a esta expansão são diversos, destacando-se a globalização, as alterações climáticas, a conjuntura socioeconómica, espécies invasoras com capacidade vetorial, urbanização de hospedeiros silvestres, bem como novos métodos de diagnóstico. Dados concretos sobre a prevalência e distribuição são cruciais para o controle de doenças animais e, no caso de *D. immitis* e *Dirofilaria repens*, também para o controlo de doenças potencialmente zoonóticas. Contudo, em Portugal, a informação existente à data de início deste trabalho era escassa e pontual. Com o intuito de clarificar esta situação e colmatar esta lacuna, foi delineado o presente trabalho para caracterizar a situação da dirofilariose e da angiostrongilose em Portugal.

Como primeiro objetivo pretendeu-se avaliar a prevalência atual e áreas de distribuição de *Dirofilaria* spp. e *A. vasorum* em canídeos domésticos e selvagens em Portugal. Relativamente à dirofilariose, foram testados 696 canídeos domésticos de três distritos do centro de Portugal (Coimbra, Santarém e Setúbal), durante 2011, 2012 e 2013. Para avaliação das espécies de *Dirofilaria* circulantes, foram utilizadas técnicas parasitológicas diretas, serológicas e moleculares. Setúbal registou a maior prevalência (24,8%), seguido de Coimbra (13,8%) e Santarém (13,2%), observando-se uma prevalência média global de 15,1%. A espécie *D. immitis* foi detetada nos três distritos, durante os três anos de estudo, com uma prevalência crescente. Em 2014, dada a escassez de dados epidemiológicos nas regiões transfronteiriças de Portugal e Espanha, este estudo foi alargado a mais sete distritos (i.e., Beja, Bragança, Castelo Branco, Évora, Faro, Guarda e Portalegre), envolvendo mais 248 canídeos. Beja foi o distrito que registou maior número de casos de infeção por *D. immitis* (8.9%), seguido pela Guarda (6,7%), Faro (2,7%) e Castelo Branco (2,5%). Não foram registados casos positivos de infeção por *D. immitis* em Bragança, Évora e Portalegre, e não foram observadas outras espécies de *Dirofilaria* em todo o País. No total observou-se uma prevalência global de 11,9% de cães positivos a *D. immitis* nos dez distritos, um valor superior comparativamente ao reportado anteriormente.

No que respeita à angiostrongilose, foi efetuado o primeiro rastreio serológico nacional de infeção por *A. vasorum*. Foram testados 906 canídeos domésticos provenientes de 16 distritos (i.e., Beja, Braga, Bragança, Castelo Branco, Coimbra, Évora, Faro, Guarda, Lisboa, Portalegre, Porto, Santarém, Setúbal, Viana do Castelo, Vila Real e Viseu). Foi utilizada a nova técnica de imunoabsorção enzimática (ELISA) de elevada sensibilidade para deteção de antigénios circulantes de *A. vasorum* e de anticorpos específicos contra este parasita. Observou-se um total de 0,7% de animais positivos simultaneamente para antigénio e anticorpo, dispersos pelas zonas norte, centro e sul de Portugal, indicando a presença de infeções ativas de *A. vasorum* distribuídas pelo País. Adicionalmente observou-se 1,3% de animais positivos apenas para anticorpo de *A. vasorum*, sugerindo uma exposição prévia ao parasita. No geral, esta prevalência foi semelhante à registada noutros países europeus. Este rastreio permitiu não só confirmar a endemicidade de *A. vasorum* em cães de diferentes zonas geográficas de Portugal, como colmatar a lacuna de conhecimento deste parasita em Portugal.

Atendendo ao facto de os cães militares constituírem um grupo de risco de exposição a doenças transmitidas por vetores pela natureza da sua atividade e longos períodos de permanência no exterior, foi efetuado um rastreio de infeção por *D. immitis* e *A. vasorum* à população de canídeos da Força Aérea Portuguesa. Foram testados 100 animais, mantidos em bases militares localizadas em Aveiro, Beja, Leiria, Lisboa, Setúbal, Madeira e Açores. Dos animais testados, 5% apresentaram anticorpos específicos contra *A. vasorum* (Aveiro, Lisboa e Setúbal) e 1% antigénios circulantes de *A. vasorum*. Não foram observados casos de infeção para *D. immitis*. Este rastreio permitiu alargar a informação sobre a presença e distribuição geográfica de *D. immitis* e *A. vasorum* em Portugal.

Com o objetivo de avaliar a prevalência de *D. immitis* e *A. vasorum* em canídeos silvestres, foi efetuado um rastreio serológico a 118 raposas (*Vulpes vulpes*) abatidas durante a época oficial de caça, provenientes de oito distritos de Portugal, distribuídos por duas regiões: Norte (Aveiro, Braga, Bragança, Porto, Viana do Castelo e Vila Real) e Sul (Évora e Setúbal). Observou-se uma prevalência de 8,5% de animais positivos para *D. immitis*. Adicionalmente, foi encontrada uma prevalência de 12,7% de animais positivos para *A. vasorum*, dos quais 5,9% simultaneamente positivos para antigénios e anticorpos de *A. vasorum*, 5,1% positivos exclusivamente para antigénios de *A. vasorum* e 1,7% positivos exclusivamente para anticorpos de *A. vasorum*. Os animais positivos para dirofilariose cardiopulmonar e angiostrongilose foram detetados nas regiões Norte e Sul de Portugal, ampliando a distribuição destes agentes a nível silvestre e destacando o seu potencial papel como reservatório para carnívoros domésticos.

Embora os hospedeiros definitivos da dirofilariose sejam principalmente canídeos domésticos e selvagens, *D. immitis* apresenta baixa especificidade de hospedeiro vertebrado, sendo capaz de infetar outras espécies de mamíferos silvestres, incluindo aquáticos. Durante a execução deste trabalho, surgiu a possibilidade de rastrear uma população de pinípedes mantidos num parque oceanográfico no Algarve. Foi encontrado DNA de *D. immitis* em três espécies distintas (focas comuns, leões-marinhos Californianos e Otárias Sul-Africanas), com uma prevalência global de 43.8%, e foi pela primeira vez documentada a Otária Sul Africana (*Arctocephalus pusillus pusillus*) como hospedeiro de *D. immitis*.

Tendo em conta as múltiplas implicações da bactéria *Wolbachia* spp. na patogénese da dirofilariose, outro dos objetivos deste projeto consistiu na pesquisa deste agente em canídeos domésticos, naturalmente infetados com *D. immitis* em Portugal. Utilizando métodos de deteção molecular, foi encontrado DNA de *Wolbachia pipientis* em 52,6% de amostras testadas, tendo-se deste modo efetuado a descrição deste endosimbionte em populações caninas em Portugal.

Adicionalmente, foram utilizadas ferramentas geoespaciais para avaliação do risco de transmissão de dirofilariose em Portugal, utilizando o modelo graus-dia. Recorreu-se aos valores médios diários de temperaturas registados na última década (de 2003 a 2013), em cinco estações meteorológicas: Faro, Funchal, Lisboa, Porto e São Miguel. Consideraram-se como pré-requisitos um limiar de temperatura de 14°C, 130 unidades de desenvolvimento de *Dirofilaria* spp., e uma expetativa máxima de vida dos vetores de 30 dias. A região da Madeira foi a que registou um maior número de dias com condições compatíveis à transmissão de *D. immitis* (média de 209,9 dias/ano), seguida de Faro (175,2 dias/ano), Lisboa (163,5 dias/ano), Açores (140 dias/ano) e Porto (117,2 dias/ano). Observou-se também na última década um período médio de risco de transmissão desta parasitose de 8 meses/ano na Madeira, 6,9 meses/ano em Faro, 6,4 meses/ano em Lisboa, 5,6 meses/ano nos Açores e de 5 meses/ano no Porto.

Dada a elevada virulência de *D. immitis* e complicações tromboembólicas associadas, outro dos objetivos deste estudo consistiu na elaboração de uma nova abordagem terapêutica minimamente invasiva. Para isso, desenvolveu-se um método não traumático de remoção percutânea de vermes adultos de *D. immitis*, baseado na utilização de fio coronário para extração de espécimes de vermes da artéria pulmonar e ventrículo direito, que se revelou eficaz.

Outros dos objetivos deste trabalho consistiu em caracterizar as práticas de desparasitação e o conhecimento dos proprietários de animais de companhia sobre doenças parasitárias, tendo sido efetuado um questionário aos utentes do Hospital da Faculdade de Medicina Veterinária da Universidade de Lisboa. Observou-se que apesar da generalidade dos proprietários desparasitar

o seu animal, esta prática é efetuada a intervalos irregulares e consequentemente ineficazes: apenas 11,8% dos cães são desparasitados internamente com a periodicidade aconselhada (i.e., mínimo trimestral) e apenas 28,4% estão protegidos continuamente ao longo do ano contra artrópodes. Adicionalmente 37% refere não efetuar a recolha de dejetos dos seus cães em todos os locais públicos, contribuindo para a contaminação ambiental. O nível de conhecimento público sobre parasitas e doenças parasitárias ainda é reduzido no País, com 88% dos donos afirmando nunca ter ouviu falar de "verme do coração" e 85% de "zoonose".

Como corolário deste trabalho, foi efetuada uma caracterização da situação epidemiológica nacional da dirofilariose e da angiostrongilose cardiopulmonar em canídeos nos últimos vinte anos.

Conclui-se que apesar do risco de exposição dos carnívoros ser variável de acordo com a região geográfica de Portugal, a probabilidade de infeção por *D. immitis* e *A. vasorum* é considerável em todo o País, ainda que se observe uma prevalência de *D. immitis* muito superior à de *A. vasorum*.

Tendo em conta as atuais alterações climáticas e sociodemográficas, prevê-se um aumento do risco de infeção por estes agentes em áreas endémicas e não endémicas. Atendendo à gravidade da patologia provocada por *D. immitis* e *A. vasorum* e considerando o potencial zoonótico e impacto em saúde pública da *Dirofilaria* spp., estes dados alertam para a necessidade de sensibilização da comunidade médico-veterinária e da população em geral, para a realização de uma profilaxia anti-parasitária dirigida e regular em animais de companhia em Portugal.

Palavras-chave: *Angiostrongylus vasorum, Dirofilaria immitis*, epidemiologia, Portugal, carnívoros.

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%	Percentage
AHS	American Heartworm Society
APHS	Acid phosphatase histochemical staining
APP	Acute phase protein
BID	*bis in die*, twice a day
CK-MB	Creatine kinase-MB
CPA	Cardiopulmonary angiostrongylosis
CPD	Cardiopulmonary dirofilariosis
CRP	C-reactive protein
CT	Computed tomography
cTnI	Cardiac troponin I
CVBDs	Canine vector-borne diseases
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
DPI	Days post infection
e.g.	For example
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
g	Gravity
GDD	Growing degree-days
HRCT	High resolution computerized tomography scanning
Kg	Kilogram
L1	First-stage larvae
L2	Second-stage larvae
L3	Third-stage larvae
L4	Fourth-stage larvae
L5	Fifth-stage larvae
mg	Milligram
MKT	Modified Knott's technique
ml	Millilitre
MLs	Macrocyclic lactones
mm	Millimetre
MPI	Months post infection
MRI	Magnetic resonance imaging
ºC	Celsius
PCR	Polymerase chain reaction
per os	Oral administration
SCD	Subcutaneous dirofilariosis
SCI	Science Citation Index
UK	United Kingdom
USA	United States of America
VCS	Vena cava syndrome
vs	*Versus*
WSP	*Wolbachia* surface protein
µm	Micrometre

CHAPTER 1

Bibliographic review and research objectives

1.1 General introduction to major canine heartworms

Dirofilariosis and angiostrongylosis are highly virulent and life-threatening dog diseases, which have increasingly been reported throughout Europe, with overlapping areas of endemicity (reviewed by Traversa, Di Cesare & Conboy, 2010). Numerous factors underlie this apparent expansion, including faster and incremented global transports and trade network, with the concurrent movement or relocation of infected animals from endemic to non-endemic areas. Additionally, political and demographic changes, new urban areas, irrigated crops (ideal for mosquito breeding) may have also affected its distribution. Moreover, climate changes and urbanization of vulpine reservoir populations represent other factors contributing to the spread of these diseases (reviewed by Colwell, Dantas-Torres & Otranto, 2011; Otranto et al., 2013).
Despite improved diagnostic methods, increasing awareness and effective preventives, canine heartworms are increasingly diagnosed and becoming more prevalent in areas previously of low risk. Considering all the anthropogenic factors, coupled with climate changes currently happening, it is expected that animals' exposure to infection will increase in the future, not only in endemic areas, but also in those with suitable conditions but not yet colonized. However, there is limited data and insufficient understanding of the spread of infection to draw conclusions or to predict further range expansions. No national or international surveillance mechanisms are in place to determine the prevalence and distribution of dirofilariosis and angiostrongylosis, currently considered important emerging diseases (reviewed by Helm, Morgan, Jackson, Wotton & Bell, 2010; Simón et al., 2012; Elsheikha, Holmes, Wright, Morgan & Lacher, 2014).
In an increasingly globalized world, travel and migratory flows coupled with progressive environmental alterations and human driven factors pose a great challenge to animal and public health care, which is forced to adapt to different needs regarding early diagnosis, treatment, disease prevention and control strategies for (re)emerging diseases.

1.2 Introduction and historical perspective of the parasite *Dirofilaria* spp.

Dirofilariosis encompasses a group of parasitosis caused by species of the genus *Dirofilaria*, transmitted by Culicidae mosquitoes. The name of the Genus *Dirofilaria* is derived from the Latin words "*Dirus*" meaning cruel/horrible and "*filum*" meaning thread, in respect to its filiform appearance (reviewed by Deplazes, Eckert, Mathis, von Samson-Himmelstjerna & Zahner, 2016).

Among all the species of *Dirofilaria, Dirofilaria immitis* represents the most important one in veterinary medicine, given its virulence and increasing incidence (reviewed by Simón et al., 2012). *Dirofilaria immitis* (Leidy, 1856) (Filarioidea, Onchocercidae) is the causative agent of canine and feline cardiopulmonary dirofilariosis (CPD), also known as cardiovascular dirofilariosis or heartworm disease, and the responsible for pulmonary dirofilariosis in humans. *Dirofilaria (Nochtiella) repens* is another relevant species, responsible for subcutaneous dirofilariosis (SD) in dogs and cats, and for subcutaneous and ocular dirofilariasis in humans (reviewed by Simón et al., 2012). Domestic and wild canids and felids constitute the natural hosts of these nematodes, although infection may also occur in other mammalian species (reviewed by McCall, Genchi, Kramer, Guerrero & Venco, 2008b; Simón et al., 2012).

Despite all the advances regarding disease pathology, host-parasite relationship, pathogenesis and parasite survival mechanisms, dirofilariosis remains a priority subject of study for researchers and clinicians. Furthermore, the involvement of mosquito vectors in their life cycle makes the transmission highly susceptible to climate changes, with prevalence undergoing fast and significant fluctuations in diverse geographic regions over the last decades (reviewed by Morchón, Carretón, González-Miguel & Mellado-Hernández, 2012; Simón et al., 2012).

1.3 Epidemiology of *Dirofilaria* spp.

1.3.1 General distribution

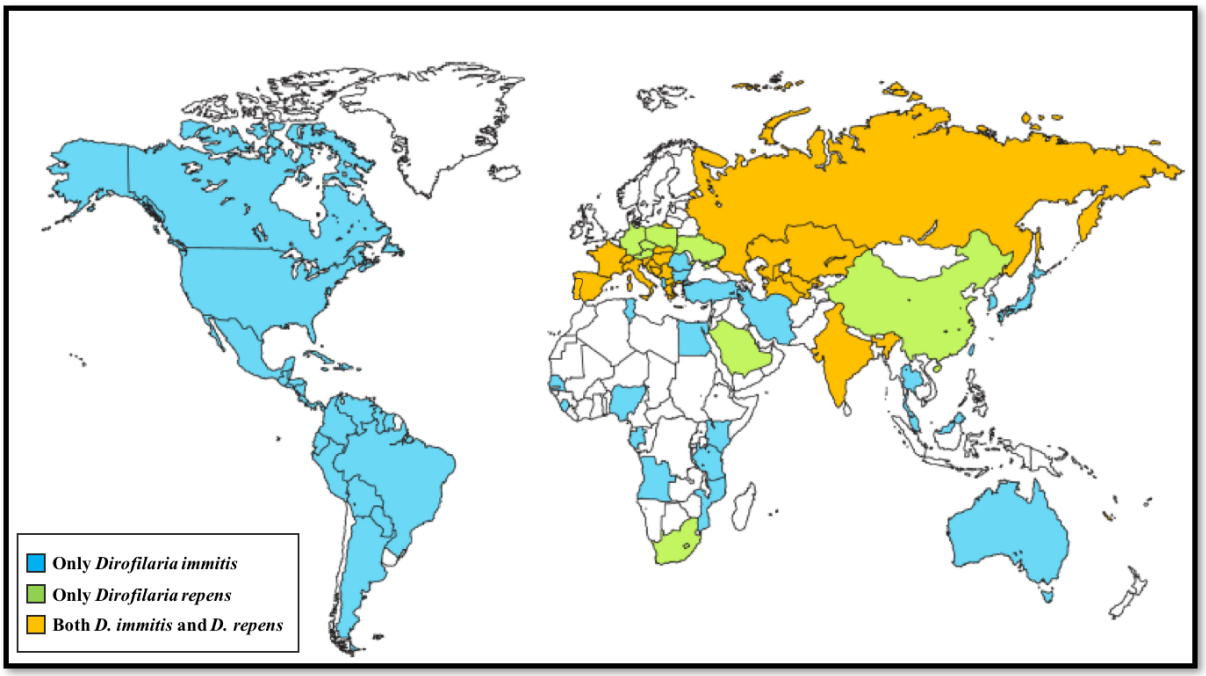
Dirofilaria immitis is present in tropical and temperate regions throughout the world, including canine populations from Africa, America, Asia, Australia and Europe (Genchi, Rinaldi, Cascone, Mortarino & Cringoli, 2005; reviewed by Simón et al., 2012). *Dirofilaria repens* is exclusive to the Old World, reported in Africa, Asia and Europe (Fig. 1). In the European continent, dirofilariosis profile is described by the presence of both *D. immitis* and *D. repens*, with some countries reporting the coexistence of both species (reviewed by European Scientific Counsel Companion Animal Parasites [ESCCAP], 2012; Simón et al., 2012). In endemic regions of southern Europe and United States of America (USA), local prevalence in dogs may reach 50%. Nevertheless, comparisons of the current and past epidemiological data show significant changes in the distribution pattern and prevalence of dirofilariosis, highlighting the establishment of new foci and increasing prevalence throughout the world (reviewed by Simón et al., 2012). Epidemiological surveys and recent clinical reports describe a significant expansion of canine autochthonous infections by *D. immitis* and/or by *D. repens,* particularly in central and northern European countries, areas where dirofilariosis was not reported or only

sporadic cases were documented (Genchi et al., 2005; Svobodova & Misonva, 2005; Duscher et al., 2009; Overgaauw & Van Dijk, 2009; Pantchev et al., 2009).

Portugal is regarded as a country where canine dirofilariosis by *D. immitis* is endemic, given its geographical situation in southern Europe, and its favourable climate for vector development, breeding and survival. According to Pereira da Fonseca, Madeira de Carvalho, Carvalho and Carvalho-Varela (1991), reviewed by Araújo (1996) dirofilariosis was prevalent in dogs from regions of southern Portugal, such as Algarve (12%), Alentejo (16.5%), Ribatejo (16.7%); regions of northern Portugal, such as Beira Litoral (4.2%) and Entre Douro e Minho (6.9%); and in Madeira Island (30%), with an overall prevalence of 14.1% microfilaremic dogs. However, this study was exclusively based on microfilariae detection, without differentiating *D. immitis* from other apathogenic or weakly pathogenic canine filarial species such as *Acanthocheilonema* (syn. *Dipetalonema*) *dracunculoides* and *Acanthocheilonema* (syn. *Dipetalonema*) *reconditum,* that are known to occur in Portugal. Additionally, in Azores, no cases of dirofilariosis were detected and no microfilaraemia was found in a survey on guard dogs (Medeiros, 1995). In a retrospective study performed in Madeira, a total of 22% dogs were microfilaremic, of which 89% of the microfilariae were histochemical identified as *D. immitis* and the remaining as *A. reconditum* and *A. dracunculoides* (Clemente, 1996). In northern Portugal, in the municipality of Alijó, 4.4% of the tested dogs were microfilaremic, of which 80% of the microfilariae were identified by acid phosphatase histochemical staining (APHS) as *A. dracunculoides*, 14% as *D. immitis* and 6% as *A. reconditum*; in the municipality of Sabrosa, 11.8% of the surveyed dogs were microfilaremic, all identified as *A. dracunculoides* (Santos, Cardoso & Rodrigues, 2000). All these studies were based exclusively on microfilariae detection, thereby underestimating occult infections.

Regarding wild canids, 3.2% red foxes (*Vulpes vulpes*) were found infected by *D. immitis* in northern-centre locations (Eira, Vingada, Torres & Miquel, 2006) and 11.8% in southern and central districts of Portugal (Carvalho-Varela & Marcos, 1993).

Figure 1 - Geographical distribution of canine dirofilariosis. Adapted from Simón et al., (2012).



1.3.2 Climate-matching model and seasonality

The transmission of *D. immitis* and *D. repens* depends on several factors, such as: sufficient numbers of infected and microfilaremic dogs, competent mosquito species and suitable climatic conditions that allow the extrinsic incubation of the parasites in the vector mosquitoes (Medlock, Barrass, Kerrod, Taylor & Leach, 2007).

Forecast models based on growing degree days (GDD) have been used to predict the occurrence and seasonality of *D. immitis* and *D. repens* in different parts of the world (Slocombe, 1989; Genchi et al., 2005; Genchi, Rinaldi, Mortarino, Genchi & Cringoli, 2009). The model is based on evidence that there is a threshold temperature of 14ºC below which *Dirofilaria* development will not occur in mosquitoes. Furthermore, there is a requirement of 130 GDD for larvae to reach infectivity and a maximum life expectancy of 30 days for mosquitoes (Lok & Knight, 1998; Slocombe, 1989). Models have allowed to estimate the number of annual *Dirofilaria* generations, as well as the length of infection risk periods in several regions of Europe (Genchi et al., 2005; 2009; Genchi, Kramer & Rivasi, 2011). Overall, the length of the transmission season is critically dependent on the accumulation of sufficient heat to incubate larvae to the infective stage in the mosquito (Lok & Knight, 1998). In the northern hemisphere, the peak months for transmission are typically July and August. Although the transmission decreases in the winter months, the risk never reaches zero, due to the presence of microenvironments in urban areas. Early predictions estimated that dirofilariosis would have the conditions to be

introduced into central and northern Europe, where it was previously not reported, which is now demonstrated (Svobodová, Svobodová, Genchi & Forejtek, 2002; Jacsó et al., 2009; Kartashev et al., 2011; Tasić-Otašević, Trenkić Božinović, Gabrielli & Genchi, 2015; Fuehrer et al., 2016). Indeed, the ongoing climate changes are lengthening the annual periods of mosquito activity and shortening larval developmental stages, with a consequent increase in the transmission in several areas.

Moreover, it's important to consider the introduction of new species of competent mosquitoes, like *Aedes albopictus* (the Asian tiger mosquito), a highly adaptable species. This vector is native from south-eastern Asia and western Pacific, but has already spread to Africa, America and Europe, becoming adapted to colder climates (Roiz, Rosà, Arnoldi & Rizzoli, 2007). Other examples of invasive species introduced in Europe are *Aedes koreicus* and *Aedes japonicus,* which are enhancing the risk of spreading *D. immitis* in endemic and non-endemic areas (Montarsi et al., 2015; Silaghi, Beck, Capelli, Montarsi & Mathis, 2017).

1.3.3. Risk factors and populations at risk

As other mosquito-borne diseases, the distribution of dirofilariosis is prevalent in regions that show high average temperatures throughout (or part of) the year and high humidity. The last condition can be provided by the existence of abundant rainfall or by the proximity to water sources, such as irrigated cultivation areas, wetlands and rice fields. Generally, the prevalence in rural and peri-urban areas is higher than in urban ecosystems. This is usually explained by a set of factors: the largest number of irrigated fields in rural areas that attract vectors and allow their proliferation; the abundant presence of wildlife animals that may carry the infection and spread the disease; the highest number of stray dogs in rural areas along with sporadic or incorrect pet chemoprophylaxis, contributing to the perpetuation of the disease.

The Atlantic islands (Madeira and the Canaries) constitute an optimal environment for the transmission of dirofilariosis as the average temperatures tend to be moderate or high, and marine environment allows high humidity rates (reviewed by Simón et al., 2012; Diosdado et al., 2016).

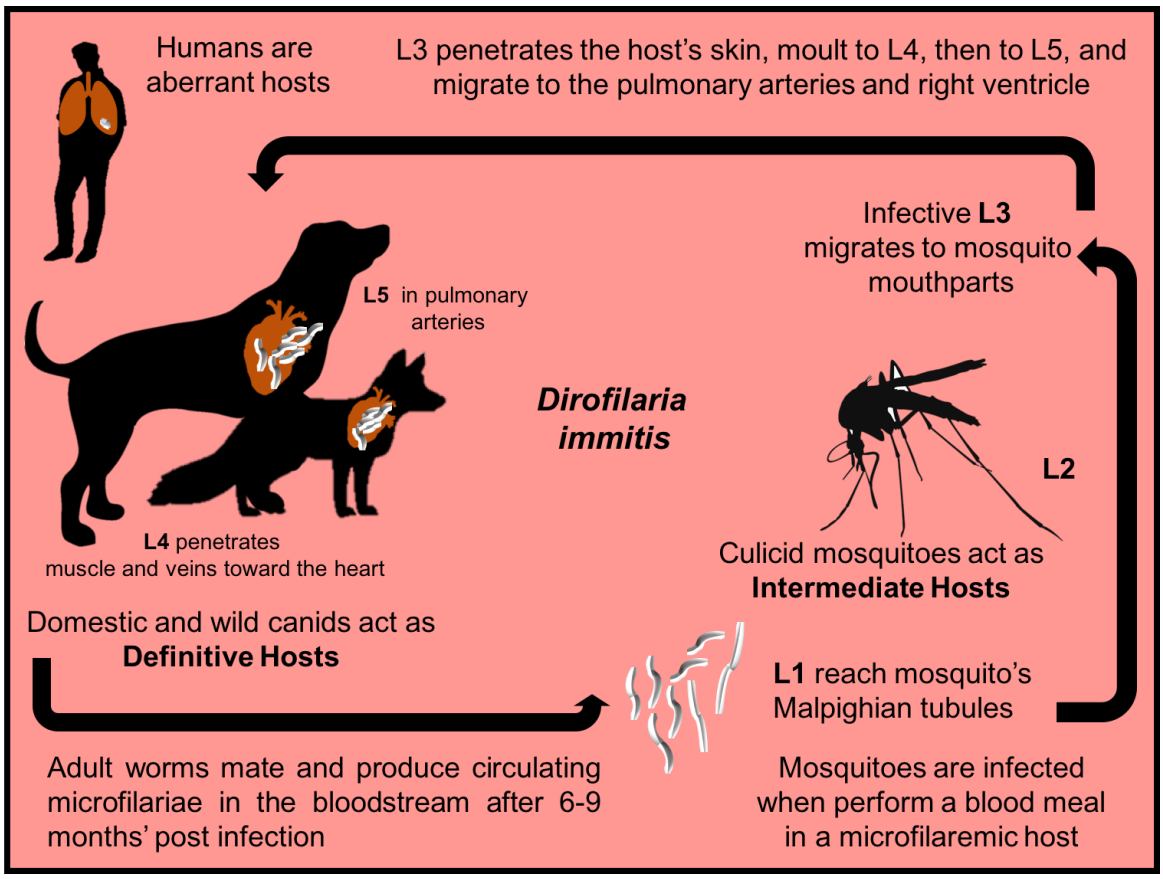
In what concerns risk populations, both outdoor and indoor pets are at risk of dirofilariosis. Nevertheless, outdoor animals or those who spend more time outside (e.g., shepherd, military, hunting or guard dogs) particularly during peak mosquito hours, in areas of high endemicity, are naturally at an increased risk of infection (reviewed by Simón et al., 2012; American Heartworm Society [AHS], 2014).

1.4 Biology of *Dirofilaria* spp.

1.4.1 Life-cycle

The life cycle of *D. immitis* and *D. repens* includes a definitive vertebrate host (domestic and wild canid as the main host of infection) and a vector (mosquitoes Diptera: Culicidae) (Cancrini & Kramer, 2001). In dogs, the life cycle of *D. immitis* is long (usually ranging from 7 to 9 months) (Kotani & Powers, 1982). It starts when a susceptible mosquito performs a blood meal on a microfilaremic host and becomes infected (Fig. 2). Approximately 24 hours after, the first-stage larvae (L1) reach the mosquito's Malpighian tubules, where they moult into second-stage larvae (L2) within 8 to 10 days' post infection (dpi), and from L2 into third-stage larvae (L3) in approximately 3 days (Taylor, 1960). Then, infective L3 migrates to the mouthparts of the vector, where they reside until the following blood meal. The time required for the development of microfilariae into the infective stage in the mosquito is temperature dependent (e.g., 10 to 14 days at 27°C and 80% of relative humidity; with cooler temperatures, the time required for the development is longer and will only progresses above 14°C) (Kartman, 1953; Slocombe, 1989). During a blood meal, infective L3 larvae (with approximately 1 mm long) migrates to the proboscis and invades the definitive host through the wound generated by the blood-feeding mosquito (Venco, Genchi & Simón, 2011). Then L3 moult to fourth-stage larvae (L4) between 3 and 12 dpi and penetrates muscle and veins toward the heart and lungs (Kotani & Powers, 1982; Lichtenfels, Pilitt, Kotani, & Powers, 1985). The subsequent moult takes place between 50-70 dpi, producing the pre-adult worms (fifth-stage larvae, L5), that arrive in the pulmonary arteries and right ventricle at between 70-85 dpi, grow and reach sexual maturity at 120 dpi. Adult *D. immitis* worms mate and produce circulating unsheathed microfilariae in the bloodstream after 6-9 months' post infection (mpi) (Kotani & Powers, 1982; McCall et al., 2008b). The concentration of microfilariae in the peripheral blood has periodicity along the day, reaching highest density values in late afternoon and early evening, in accordance with the maximum activity of the intermediate host. Microfilariae and adult worms may last in dogs for 2 and 7 years, respectively (Venco et al., 2011). Some infected dogs do not develop microfilaraemia, an event, possibly explained by single-gender worm infections and host immune response factors (Simón & Genchi, 2000).

Figure 2 - Life cycle of *Dirofilaria immitis* (original).



Regarding *D. repens,* although adult worms can be found in the abdominal cavity and muscular fasciae (Genchi et al., 2011), they usually inhabit the subcutaneous tissues of definitive hosts where they achieve sexual maturity at 6-9 mpi (Manfredi, Di Cerbo & Genchi, 2007).

Numerous species of culicid mosquitoes are involved in the transmission of *D. immitis* and *D. repens,* as revised by Cancrini and Kramer (2001) and Cancrini and Gabrielli (2007). Nearly 70 species of culicid mosquitoes, mainly belonging to the genera *Culex*, *Aedes*, *Anopheles*, *Culiseta* and *Coquillettidia* have been identified as potential vectors of animal and human dirofilariosis. However, only in few cases its real vectorial capacity has been proven. Epidemiological studies conducted on vectors in distinct continents have shown a lower prevalence of *Dirofilaria* spp. in mosquitoes in comparison to vertebrate hosts (ranging from 1% to 10%), with the higher values detected in *Culex theileri*. In Europe, *Culex pipiens* is considered the main vector of both *Dirofilaria* spp. Nevertheless, *Aedes vexans*, *Aedes punctor*, *Aedes albopictus*, *Aedes caspius* and *Anopheles maculipennis* are also species in which vector capacity has been documented in European countries (Manrique-Saide et al., 2010; Yildirim et al., 2011; reviewed by Otranto et al., 2013).

In mainland Portugal, *Culex theileri*, *Culex pipiens*, *Anopheles maculipennis* s.l., *Anopheles atroparvus*, *Aedes caspius* and *Aedes detritus* s.l. were found naturally infected with *D. immitis*

(Ferreira et al., 2015). This is worrying if we consider that out of the 41 species of mosquitoes identified in continental Portugal (Ribeiro, Ramos, Pires & Capela, 1988), *An. maculipennis* s.l., *Cx. pipiens* s.l., *Cx. theileri* and *Ae. caspius* were the most abundant and broadly distributed (Almeida et al., 2008) and are all proven vectors of *D. immitis*.

1.4.2 Host range and specificity

CPD and SCD primarily affect members of the family Canidae, representing its main reservoir of infection. Nevertheless, *D. immitis* and *D. repens* demonstrate poor vertebrate host specificity, being able to infect several mammalian species, such as black bears, California sea lions, domestic cats, polecats, cougars, ferrets, jaguars, leopards, lions, seals, ocelots and tigers. Humans may also be infected, representing aberrant hosts. From an epidemiological point of view, cats are considered to play a minor role in the transmission cycle of dirofilariosis as its microfilaraemia is low and of short duration (Barriga, 1982; McCall et al., 2008b).

1.4.3 Role of wildlife hosts

Several species of wild carnivores were found infected by *D. immitis* and *D. repens,* including coyotes (*Canis latrans*), dingoes (*Canis lupus dingo*), red (*Vulpes vulpes*) and grey (*Urocyon cinereoargenteus*) foxes, jackals (*Canis aureus*) and wolves (*Canis lupus lupus*), with variable prevalences (Franson, Jorgenson & Boggess, 1976; Marconcini, Magi, Macchioni & Sassetti, 1996; Mañas, Ferrer, Castellà & López-Martín, 2005; Magi et al., 2008; Cirović et al., 2014; Gavrilović, Blitva-Robertson, Özvegy, Kiskároly & Becskei, 2014; Ionică et al., 2016). Indeed, carnivores with peridomestic habits are known to constitute an excellent sentinel for the spread of *D. immitis* (Sacks & Caswell-Chen, 2003; Wixcom, Green, Corwin & Fritzell, 1991).

1.4.4 Morphological characteristics of *Dirofilaria immitis* and *Dirofilaria repens*

Dirofilaria immitis adult worms are threadlike nematodes, presenting six small papillae surrounding the mouth opening, with females measuring 250-300 mm in length and 1-1.3 mm in diameter and males measuring 120-200 mm in length and 0.7-0.9 mm in diameter (Manfredi et al., 2007). *D. immitis* microfilariae (after fixation with 2% formalin) measure 290-330 μm in length and 5-7 μm in diameter, and have a straight posterior end and a conical-shaped cephalic extremity.

Dirofilaria repens adult worms are smaller than *D. immitis*, with females measuring 100-170 mm in length and 4.6-6.3 mm in diameter, and males measuring 50-70 mm in length and 3.7-4.5 mm in diameter (Manfredi et al., 2007). *D. repens* microfilariae also reside in the bloodstream. Nonetheless, they are longer, measuring between 350-385 μm in length and 7-8 μm in diameter (after fixation with 2% formalin), with a hook-shaped tail and a blunt cephalic extremity (Venco et al., 2011).

1.4.5 Role of the symbiotic bacteria *Wolbachia* spp.

Both *D. immitis* and *D. repens* host a symbiotic intracellular bacterium that belongs to the order *Rickettsiales*, genus *Wolbachia* (Sironi et al., 1995). This bacterium is found in all filarial developmental stages, i.e., located in the genital organs of females and on the lateral cords of both males and females, as well as in microfilariae and in the larvae in the vector. As *Wolbachia* spp. are involved in the moulting and embryogenesis of filariae (Bandi, Dunn, Hurst & Rigaud, 2001), these bacteria are vital for the development of larvae and for the survival of adult worms in vertebrate hosts (McGarry, Egerton & Taylor, 2004). Indeed, in the 1990s, it was shown that tetracycline treatment of filarial-infected animals led to the interruption of worm development in the host, disruption of microfilarial production and long-term survival of adults (reviewed by McCall et al., 2008a). *Wolbachia* spp. is also an important player in the interaction with the immune system of the infected host, associated with the upregulation of pro-inflammatory cytokines, neutrophil recruitment and increasing specific immunoglobulins (Taylor, Bandi & Hoerauf, 2005). This ground-breaking discovery resulted in a profound shift regarding a better understanding of the filarial biology, including the pathological mechanisms caused on the hosts, but most particularly, on the treatment approach, with the valuable association of an antibiotic in the treatment of dirofilariosis (reviewed by Simón et al., 2012).

1.5 Pathophysiology and clinical signs of *Dirofilaria* spp.

Canine CPD is a life-threatening disease caused by the presence of *D. immitis* adult worms (Furlanello, Caldin, Vezzoni, Venco & Kitagawa, 1998; McCall et al., 2008b) and by their antigenic products (Kramer, Simón, Tamarozzi, Genchi & Bazzocchi, 2005). It has a chronic progression, with numerous dogs showing no clinical signs for months or years, unless there is a large worm burden (until 250 worms) and/or animals undergo strenuous exercise.

1.5.1 Respiratory and cardiovascular signs caused by *Dirofilaria immitis*

The main location of *D. immitis* adult worms is in the pulmonary arteries followed by the right ventricle (Fig. 3), so the first effects occur in the pulmonary arteries and lungs and, secondarily, in the heart. Clinical signs develop gradually, often beginning with a chronic unproductive cough (that increases with exercise), later associated with dyspnoea, regurgitation and lethargy. Infected dogs may also exhibit intolerance to exercise or syncope related with increased physical activity or excitement. Physical examination may reveal evidence of weight loss, right-sided heart murmur of tricuspid insufficiency, split-second heart sound and cardiac gallop (reviewed by Atkins, 2010). Whenever right heart failure is present, jugular venous ingurgitation and pulsation, along with hepatosplenomegaly and ascites may occur. Pulmonary manifestations include cough, dyspnoea, pulmonary crackles, muffled lung sounds and eventually cyanosis. If pulmonary thromboembolism occurs, dyspnoea may worsen and fever and haemoptysis may be noted. Although sudden death is rare, it can occur due to cardiorespiratory insufficiency or severe thromboembolism (Venco et al., 2011).

Figure 3 - Adult nematodes of *Dirofilaria immitis* collected at the necropsy of a pinniped (left) and a domestic dog (right) (original).

1.5.2 Other clinical signs caused by *Dirofilaria immitis*

Dirofilaria immitis may cause renal dysfunction and glomerulonephritis induced by the deposition of immune complexes triggered by the antigens of adult worms and larval stages (Abramowsky, Powers, Aikawa & Swinehart, 1981; Paes-de-Almeida, Ferreira, Labarthe, Caldas & Mc-Call, 2003). Dogs may also develop eczematous dermatitis (secondary to kidney disorders) and eosinophilic pneumonia, due to an eosinophilic reaction against microfilarial antigens. Other organs may also be affected by ectopic localisations, such as the eyes, brain, liver and peritoneal cavity, with related pathologies.

1.5.3 Clinical signs caused by *Dirofilaria repens*

SD is usually subclinical, although manifestations may occur associated with the presence of adult worms of *D. repens* between subcutaneous and deep connective tissue layers. The primary clinical sign is the presence of one or more skin non-inflammatory nodule(s), ranging from 0.5–3 cm, located in different anatomical sites. Other manifestations include pruritus, erythema, papules, focal or multifocal alopecia and hyperkeratosis (Tarello, 2011). Extra-dermic signs may occur, such as anorexia, vomiting, lethargy, fever, lymphadenomegaly and conjunctivitis. Intra-vitreous infection is rare in dogs, but may be caused by *D. repens* (Guterbock, Vestre & Tood, 1981).

1.6 Diagnosis of canine dirofilariosis

The diagnosis of *Dirofilaria* infections may be done through the microscopic detection and morphological identification of circulating blood microfilariae; by the detection of circulating adult worm antigens and/or antibodies (only available for *D. immitis*) in serum; or by the detection of DNA of *Dirofilaria* using molecular methods.

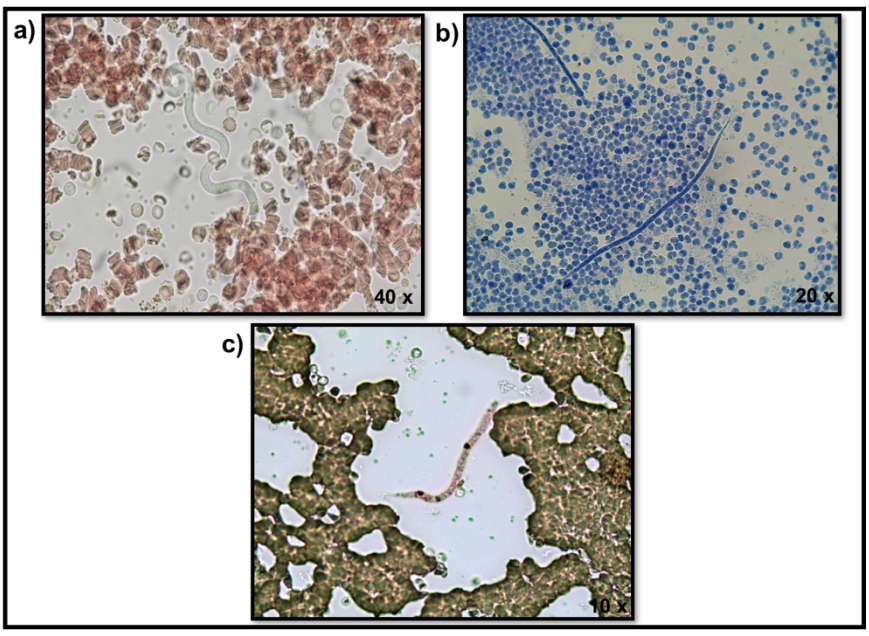
1.6.1 Parasitological diagnosis

The most commonly used method for microfilariae identification is the Modified Knott's technique (MKT) (Fig. 4). It is an easy, quick and inexpensive diagnostic concentration method. Briefly, 1 ml of EDTA blood is mixed with 9 ml of 2% formalin and centrifuged for 5 minutes at 500×g. After that, the supernatant is poured off, one drop of blue methylene is added and the sediment is observed under the light microscope (Venco et al., 2011; Magnis et al., 2013). Given the variety of canine filarial species presenting blood microfilariae [(with *D. immitis*, *D.*

repens, *A. dracunculoides* and *A. reconditum* as the most important in Europe (Genchi et al., 2011)] it is essential to perform a morphometric analysis to obtain a correct diagnosis and select the appropriate treatment. Magnis et al., (2013) validated morphometric criteria for the identification of microfilariae in the dog's blood using the MKT, allowing a clear distinction between *D. immitis* (302 µm average length, 6 µm average width, with a conical front end and a straight rear end), *D. repens* (369 µm average length, 9 µm average width, with a conical front end and curved caudal end), *A. dracunculoides* (259 µm average length, 5 µm average width, with a round front and straight caudal end) and *A. reconditum* (265 µm average length, 5 µm average width, with a blunt front end and a small hook in the rear end). Due to the overlapping size ranges of *A. dracunculoides* and *A. reconditum*, biochemical or molecular methods are required to distinguish these two species (Magnis et al., 2013).

Acid phosphatase histochemical staining is an additional method that allows the distinction of microfilariae according to their anatomical regions according to the acid phosphatase activity (Chalifoux & Hunt, 1971). Nowadays this method is inclusively available as a commercial kit (Peribáñez et al., 2001). *Dirofilaria immitis* microfilariae has two acid phosphatase activity zones (near the anal and excretory pores) (Fig. 4c), whereas *D. repens* has only one (near the anal pore and eventually some inner body complex). Other canine filariae, such as *A. dracunculoides* exhibits three areas of enzymatic activity (anal pore, excretory pore and internal body in-between these two pores) and *A. reconditum* exhibits a diffuse staining (Chalifoux & Hunt, 1971; Balbo & Abate, 1972).

Figure 4 - Microfilariae of *Dirofilaria immitis* in: a) fresh blood smear; b) stained by the Modified Knott's technique; c) stained with the acid phosphatase, highlighting the anal and excretory pores (original).



1.6.2 Immunological diagnosis

Serological methods are other useful diagnostic techniques that allow the detection of amicrofilaremic infections, not detectable by MKT or APHS. Highly specific and sensitive enzyme linked immunosorbent assays (ELISA) or immunochromatography-based assays that detect circulating adult worm antigens of *D. immitis* females are commercially available for the diagnosis of cardiopulmonary dirofilariosis (Simón, Genchi, Prieto & Allende, 2001; Venco et al., 2011). However, commercial antigen-detection kits for *D. immitis* were tested with sera from dogs infected with *A. vasorum*, and cross-reactions were detected (Schnyder & Deplazes, 2012), which is of some concern.

1.6.3 Molecular diagnosis

Other diagnostic options are the amplification of microfilaria DNA by PCR (Favia, Lanfrancotti, della Torre, Cancrini & Coluzzi, 1997a; Favia Tringali, & Cancrini, 1997b). A duplex real-time PCR was developed to detect and differentiate infection by *D. immitis* and *D. repens* in dogs and mosquitoes and a multiplex PCR was described for the simultaneous detection of canine filarioids (Latrofa et al., 2012a,b).

1.6.4 Laboratory diagnosis

Marked alterations in haematology and biochemistry are usually seen when acute changes take place or in the late stage of the disease. Leukocytosis, eosinophilia, neutrophilia and non-regenerative normocytic normochromic anaemia is frequently seen. Thrombocytopenia can also be found, particularly when disseminated intravascular coagulation (DIC) is present, as well as azotaemia and hyperbilirubinemia. An increase in the acute phase protein (APPs), such as C-reactive protein (CRP) occurs and is useful for staging the disease and monitoring recovery after treatment. The concentration of cardiac troponin I (cTnI), myoglobin and creatine kinase MB (CK-MB) increase in dogs with high parasite burden, due to myocardial injury, and may also be used as early markers of cardiac damage. Similarly, D-dimer concentrations increase after thromboembolism, being useful on the diagnosis of pulmonary thromboembolism in dogs (Carretón et al., 2011).

1.6.5 Imaging findings

Thoracic radiograph, echocardiography and electrocardiography provide insights regarding the clinical status, severity and prognosis of cardiopulmonary disease secondary to heartworm infection. Characteristic (nearly pathognomonic) radiographic features are enlarged, tortuous, truncated peripheral intralobar and interlobar branches of the pulmonary arteries (particularly on the diaphragmatic lobes), accompanied by pulmonary parenchymal disease, right heart cardiomegaly in advanced stages, and pleural effusion following right heart congestive failure (Rawlings, 1986; Bowman & Atkins, 2009). Echocardiography allows the visualization of the worms, which are seen as two parallel hyperechoic lines in the main pulmonary artery, interlobe branches, right heart atrium and ventricle (Badertscher, Losonsky, Paul & Kneller, 1988). Besides, allows the assessment of cardiac anatomy and functional capacity, providing conclusive confirmation of the vena cava syndrome (VCS) when heartworms are located in the tricuspid valve. However, echocardiography is not an efficient method of making this diagnosis, particularly in lightly infected dogs, as the worms are frequently limited to the peripheral branches of the pulmonary arteries, thus beyond the echocardiographic field of view.
Electrocardiography can reveal alterations in the electrical axis and rhythm (deviations to the right side of the axis and atrial fibrillation) in dogs in terminal stages that exhibit severe enlargement of the right atrium (Venco et al., 2011).

1.6.6 Pathological findings

The first lesions occur on the walls of the pulmonary arteries and will lead to the development of subsequent pulmonary and cardiac pathology, observed in the last stage of infection (Venco, 2007). Worms induce mechanical trauma in the pulmonary arteries, causing a proliferative pulmonary endarteritis, with intravascular villus formation. Additionally, smooth muscle proliferation and consequent lumen narrowing and reduction of their compliance occur. All these changes in the arterial walls coupled with the release of inflammatory mediators lead to pulmonary hypertension. These events generate an overload on the right side of the heart, culminating in *cor pulmonale*, congestive right heart insufficiency and consequent hypertrophy and dilation. If congestive heart failure develops, venous ingurgitation, peripheral oedema, hydrothorax, hydropericardium and ascites may occur. Hepatomegaly may also happen, leading to liver insufficiency, jaundice and coagulation disorders. Additionally, worms' death may cause a severe inflammation that could induce thromboembolism (Calvert & Rawlings, 1985; Rawlings, 1986). An acute and fulminant multisystemic condition that may also develop is

VCS, predominantly occurring in small dogs. This happens when a mass of worms displaces from the pulmonary arteries to the right ventricle, blocking the tricuspid valve and circulating blood, causing an overload of the right atrium and the caudal vena cava. This leads to the death of the animal due to hemolysis and DIC, other severe multisystemic condition (Furlanello et al., 1998).

1.6.7 Difficulties and misconceptions in diagnosing canine dirofilariosis

Although filarial species can be identified by microfilarial size and morphology, these features are challenging, particularly when low parasitaemia or mixed infections are present. Besides, both MKT and APHS only work when heartworm-infected dogs have a detectable microfilaraemia. This happens only in 2/3 of the cases, as 30-40% of infected dogs remain or become amicrofilaremic despite a persisting infection with adults (reviewed by Deplazes et al., 2016). Moreover, APHS reagents have a limited shelf life and require fresh samples to yield interpretable results (Peribáñez et al., 2001).

Diagnoses exclusively based on circulating antigen, may give a false negative result in infections with low parasite burden. Furthermore, in some dogs, antigen–antibody complexes may entrap antigens, hampering the immunological detection. As circulating antigens are only detectable when *D. immitis* reached the adult stage, antigen testing should not be carried out earlier than 7 months after exposure to infection. For *D. repens*, there are still no available immunologic methods.

The combination of the serological techniques with MKT or APHS allows an accurate detection of dirofilariosis. A positive microfilaria test with a positive antigen test confirms an infection with *D. immitis*. A positive antigen test without circulating microfilariae indicates an amicrofilaremic or occult infection by *D. immitis* that may be due to pre-patency, unisex infection by female worms, drug-induced sterility of adult filariae, or even immune-mediated clearance of microfilariae (Genchi, Venco & Genchi, 2007). A positive microfilaria test with a negative antigen test may indicate an infection caused by a species apart from *D. immitis* that may be confirmed by MKT or molecular techniques. If it is *D. immitis* microfilariae, it might be due to antigen-antibody complex formation that can interfere with the antigen detection (Little et al., 2014), low female worm burden, or the persistence of microfilariae following the natural or the pharmacological death of adults (Atkins, 2003).

Overall, diagnostic techniques must be taken together (testing for both microfilaria and antigens) and interpreted along with the results from clinical examination (thoracic radiography and echocardiography) to achieve a reliable diagnosis. Annual testing of dogs is important not

only to ensure that prophylaxis is correctly being performed but also to provide early treatment with lower pathological effects when an infection is present (AHS, 2014).

1.7 Treatment of canine dirofilariosis

The main goal of *D. immitis* treatment is to eliminate all forms of the parasite (e.g., adults and larval stages) and improve the animal's clinical conditions and welfare, with minimal complications (AHS, 2014). This can be achieved pharmacologically using a multimodal approach, combining melarsomine dihydrochloride (an adulticide drug) with macrocyclic lactones (MLs) (microfilaricide) and doxycycline (antibiotic against *Wolbachia* spp. organisms). Mechanical heartworm removal is also indicated as a method of eliminating as many adult worms as possible before pharmacological treatment is initiated (reviewed by Nelson, 2012; AHS, 2014). Despites the range of therapeutic options, it's important to underline the complexity and inherent risk associated with the treatment of cardiopulmonary dirofilariosis, given the massive worm destruction in the bloodstream and its multiple side effects (reviewed by Simón et al., 2012). Before starting therapy, the staging and risk of thromboembolic complications of each animal should be assessed, considering age, dog size, parasite load, severity of pulmonary disease and possibility to restrict dog's physical activity (Venco, McCall, Guerrero & Genchi, 2004).

Treatment of SD is indicated in dogs suffering from clinical signs (e.g., dermal swelling, nodules, pruritus) and to decrease the risk of infection to other dogs and humans near the infected animal. Worms of *D. repens* can also be removed from the subcutaneous nodules. Additionally, imidacloprid 10% combined with moxidectin 2.5% (spot-on) has proven to be effective against adult *D. repens* worms and microfilariae, with monthly treatments (Petry et al., 2015).

1.7.1 Anthelmintic therapy

The only adulticidal drug available and approved by the Food and Drug Administration (FDA) for *D. immitis* is melarsomine dihydrochloride, an arsenic compound. According to the American Heartworm Society (AHS), the three-dose protocol of melarsomine (one injection of 2.5 mg/kg followed one month later by two injections of 2.5 mg/kg, 24 hours apart) is the recommended regimen regardless the stage or severity of the disease (with the exception of VCS) (Table 1). The three-dose protocol has proven to have an overall increased safety and efficacy (98% *vs* 90%) in comparison to the two-injection protocol (two injections of 2.5 mg/kg,

24 hours apart) (AHS, 2014). Strict exercise restriction for 30 to 40 days after adulticide treatment is crucial for minimizing cardiopulmonary complications, such as pulmonary thromboembolisms, which constitute an inevitable consequence of successful adulticide therapy. Whereas mild embolism in healthy lung areas may be clinically unapparent, severe embolism may cause life-threatening respiratory distress. Signs of embolism are usually evident within 7 to 10 days after completion of adulticide administration and may include low fever, cough, haemoptysis and exacerbation of right heart failure (Hirano, Kitagawa & Sasaki, 1992). As melarsomine has incomplete efficacy against young adult worms (less than 4 months old) (Dzimianski, McTier, McCall & Raynaud, 1989; Dzimianski, McCall, McTier & Raynaud, 1990), MLs should be administered monthly to eliminate existing larvae. As MLs may cause a rapid decrease in microfilariae numbers, it should be used with caution in dogs with high microfilarial counts, coupled with antihistamines and corticosteroids to minimize potential reactions. Treatment approaches using only MLs as a slow-kill adulticide are not recommended) (AHS, 2014).

The administration of a tetracycline antibiotic is useful to reduce the number of *Wolbachia* organisms and their metabolites such as the *Wolbachia* surface protein (WSP), a major responsible for the pathogenesis of filarial diseases. Doxycycline (at 10 mg/kg, BID, for 4 weeks) should be given before the administration of melarsomine so that *Wolbachia* organisms and metabolites are reduced or absent when the worms die and fragment. Indeed, pre-treatment with ivermectin and doxycycline prior to melarsomine in experimentally infected *D. immitis* dogs, had shown to reduce pulmonary pathology associated with the death of the heartworms (reviewed by McCall et al., 2008a; Kramer et al., 2011).

Table 1- Recommended treatment and management protocol for *Dirofilaria immitis* infections in dogs (American Heartworm Society [AHS], 2014)

Day	Treatment
Day 0	Dog diagnosed and verified as heartworm positive: • Positive antigen (Ag) test verified with microfilaria (MF) test • If no microfilariae are detected, confirm with 2nd Ag test from a different manufacturer Begin exercise restriction. • The more pronounced the signs, the stricter the exercise restriction If the dog is symptomatic: • Stabilize with appropriate therapy and nursing care • Prednisone prescribed at 0.5 mg/kg BID 1st week, 0.5 mg/kg SID 2nd week, 0.5 mg/kg EOD 3rd and 4th weeks
Day 1	Administer heartworm preventive. • If microfilariae are detected, pretreat with antihistamine and glucocorticosteroid, if not already on prednisone, to reduce risk of anaphylaxis • Observe for at least 8 hours for signs of reaction
Days 1–28	Administer doxycycline 10 mg/kg BID for 4 weeks. • Reduces pathology associated with dead heartworms • Disrupts heartworm transmission
Day 30	Administer heartworm preventive.
Day 60	Administer heartworm preventive. First melarsomine injection 2.5 mg/kg intramuscularly (IM) Prescribe prednisone 0.5 mg/kg BID 1st week, 0.5 mg/kg SID 2nd week, 0.5 mg/kg EOD 3rd and 4th weeks. Decrease activity level even further. • Cage restriction/on leash when using yard
Day 90	Administer heartworm preventive. Second melarsomine injection 2.5 mg/kg IM
Day 91	Third melarsomine injection 2.5 mg/kg IM Prescribe prednisone 0.5 mg/kg BID 1st week, 0.5 mg/kg SID 2nd week, 0.5 mg/kg EOD 3rd and 4th weeks. Continue exercise restriction for 6 to 8 weeks following last melarsomine injections.
Day 120	Test for presence of microfilariae. • If positive treat with a microfilaricide and retest in 4 weeks Establish year-round heartworm prevention.
Day 271	Antigen test 6 months after completion; screen for microfilariae.

1.7.2 Supportive therapy

Supportive therapy is indicated in dogs before receiving adulticide or surgical therapy for thrombosis prophylaxis, as well as on those in which causal therapy is not recommended. Corticosteroids, diuretics, vasodilators, positive inotropic agents and fluid therapy might be used (AHS, 2014).

1.7.3 Surgical intervention for *Dirofilaria immitis* worm's extraction

Surgical therapy is advised in dogs severely infected with *D. immitis*. These dogs are poor candidates for immediate melarsomine treatment because of the immune reaction triggered by the rapid killing of the worms that can culminate in pulmonary thromboembolism. Surgical therapy is also indicated in the case of VCS, a life-threatening condition that ends fatally within 2 days if worm's extraction is not pursued promptly (AHS, 2014).

Surgical removal can be accomplished using distinct devices, including rigid or flexible alligator forceps (Ishihara, Kitagawa & Sasaki, 1988) or intravascular retrieval snares (Yoon, Choi, Lee & Hyun, 2013). These devices are introduced via the right external jugular vein, aided by fluoroscopic guidance, to access the right cardiac chambers and the major pulmonary arteries. Unlike adulticide treatments, surgical removal of filariae can potentially avoid the risk of pulmonary thromboembolism. The principal advantages are the shorter duration of general anaesthesia and the reduced invasiveness of the procedure with lower damage of the vascular endothelium. Generally, the intraoperative mortality risk is low, and survival and recovery rates are positively correlated with the number of parasites removed (Morini, Venco, Fagioli & Genchi, 1998). After few weeks following surgery, chemotherapy is recommended to eliminate any remaining worms (AHS, 2014).

1.7.4 Prognosis

The prognosis of subclinical infected dogs with CPD is generally good. Although the prognosis for severely infected dogs should be guarded, a large number can be successfully managed. Nevertheless, dogs with severe DIC, VCS, massive embolization, pulmonary eosinophilic granulomatosis, severe pulmonary artery disease or heart failure, have a poor prognosis (Rawlings, 1986).

The prognosis of *D. repens* is generally good and dependent on the damage caused by the worms and the degree of success of surgical extraction (Dantas-Torres et al., 2009).

1.8 Control and prevention of *Dirofilaria* spp. infections

Given the severity and the therapeutic risks associated with *Dirofilaria* infection, preventative chemoprophylaxis is crucial. The most safety and effective prophylactic option is the administration of LM such as ivermectin, milbemycin oxime, moxidectin or selamectin. Although, LM do not prevent the inoculation of larvae, they impede larval development in the

vertebrate host. Prevention through injectable long lasting formulation or monthly oral or spot-on administration should start one month prior the mosquito season (early spring), and should be continued until one month after this period ends (late autumn). However, in endemic areas and regions where the climate conditions allow transmission throughout the year, continuous annual protection against heartworm is recommended (reviewed by Simón et al., 2012).

Control measures to prevent mosquitoes are also crucial to reduce *D. immitis* infection, namely: regular application of mosquito repellents, emptying standing water collections, installation of window screens and avoidance of areas and day periods in which mosquitoes are most active (ESCCAP, 2012; AHS, 2014).

For *D. repens*, the sustained release formulation of moxidectin microspheres have shown full efficacy in experimental studies (Genchi, Pengo & Genchi, 2010).

1.9 Public health relevance of *Dirofilaria immitis* and *Dirofilaria repens*

Human dirofilariasis is currently considered an emerging disease in some areas of the globe, due to the high increase of subcutaneous and ocular cases reported in the recent years, contradicting the idea that human dirofilariasis is infrequent (Kramer et al., 2007; reviewed by Simón et al., 2012). Human pulmonary dirofilariasis, usually associated with *D. immitis,* is characterized by pulmonary nodules triggered by an inflammatory response around the immature worms that reach the pulmonary artery. The most frequent presentation is a single spherical or ovoid nodule located in peripheral areas like the subpleural region. Infection is usually asymptomatic, although cough, thoracic pain, haemoptysis, dyspnoea, fever and malaise have been reported (Muro & Cordero, 2001). These nodules are frequently misdiagnosed with malignant lesions (Simón, López-Belmonte, Marcos-Atxutegi, Morchón & Martín-Pacho, 2005).

Subcutaneous dirofilariasis is characterized by firm and erythematous nodules caused by the presence of adult or pre-adult *D. repens* worms in the subcutaneous tissues. *D. repens* worms can also reach the ocular region (orbital zone, eyelids, subconjunctival and intra-vitreous tissues) (Paes-de-Almeida et al., 2003) causing ocular dirofilariasis, a serious condition that could lead to permanent complications, such as retinal detachment and glaucoma, with losses of visual acuity reported in 10% of the patients (Avdiukhina, Lysenko, Supriaga & Postnova, 1996).

Of note, it is not infrequent to find worms of both species in anatomical locations distinct from those commonly associated with each species (Pampiglione & Rivasi, 2000; Genchi et al., 2011). Thereby the automatic connotation between the location of the nodule and the potential

causative species is insufficient to ascertain the causative *Dirofilaria* species in each case (reviewed by Simón et al., 2012).

Wherever canine dirofilariosis exists, there is a risk of human infection. Nevertheless, the worldwide distribution of dirofilariasis in humans does not exactly coincide with the one reported in canids, possibly due to the lack of data in several geographical areas. Overall, approximately 1.782 cases of human dirofilariasis have been reported, of which 1.410 were subcutaneous/ocular and 372 were pulmonary (reviewed by Simón et al., 2012). In America predominates pulmonary dirofilariasis by *D. immitis*. In the Old World where both *D. immitis* and *D. repens* circulate, subcutaneous and ocular dirofilariasis by *D. repens* largely predominate over pulmonary dirofilariasis (Bartokova, Poliakova & Ermolenko, 2011), even in areas where *D. immitis* is highly endemic (Simón et al., 2005). Specifically, 586 subcutaneous/ocular and 33 pulmonary cases have been reported in the European Union, most of them originated in the Mediterranean countries where *Dirofilaria* spp. are traditionally endemic (France, Greece, Italy and Spain) (Muro, Genchi, Cordero & Simón, 1999; Pampiglione & Rivasi, 2000). However, in the last decade, the geographical range of human dirofilariasis seems to have expanded, with numerous cases reported from central and northern Europe (Genchi et al., 2005; Simón et al., 2005; Simón, Morchón, González-Miguel, Marcos-Atxutegi & Siles-Lucas, 2009). In Portugal, few data is available. Two cases of pulmonary nodules by *D. immitis* were reported (reviewed by Araújo, 1996) as well as two cases of dirofilariasis by *D. repens* (Rombert Nunes, Azevedo & Sinari, 1992; Baptista-Fernandes et al., 2015), the last one probably imported.

Despite the number of reported cases of human dirofilariasis, it's important to bear in mind that this disease is still underdiagnosed. Endemic regions with zoo-anthropophilic vectors have possibly higher frequencies of human infections than those reported in the literature, probably merely portraying "the tip of the iceberg" (Simón et al., 2005). This underreporting has been related to several factors, such as the lack of awareness and vigilance amongst health professionals, the difficulties inherent in parasite identification or the fact that symptoms in dirofilariasis patients (especially in pulmonary infections) may go unnoticed. Indeed, seroepidemiological studies conducted in endemic areas of canine dirofilariosis with antibody detection, showed rates of infection in humans like those found in canine reservoirs (Simón, Muro-Alvarez, Cordero-Sánchez & Martín-Martín, 1991; Prieto, Cancrini, Muro, Genchi & Simón, 2000; Montoya-Alonso et al., 2010). This is important as it suggests that the risk of human infection by *D. immitis* and *D. repens* is probably higher than the number of reported cases.

2.1 Introduction and historical perspective of the parasite *Angiostrongylus vasorum*

Canine cardiopulmonary angiostrongylosis (CPA) is a severe disease caused by the metastrongyloid nematode *Angiostrongylus vasorum* (Baillet, 1866) Kamensky, 1905 (Nematode: Metastrongylidae). The pseudonym "French heartworm" was given since it was firstly recognized in France by Serres (1854). The Family name Angiostrongylidae is derived from the Greek words "Angeion" meaning vessel and "strongylos" meaning round. Even after its discovery over a century ago, angiostrongylosis remains a high priority for clinicians and researchers due to its severity, increased incidence and spread into previously uninfected areas (reviewed by Elsheikha et al., 2014).

2.2 Epidemiology of *Angiostrongylus vasorum*

2.2.1 General distribution

Angiostrongylus vasorum has been characterized by a patchy distribution, with geographic areas of high prevalence (hotspots), surrounded by regions with occasional occurrence or where it has never been recorded (Morgan, Jefferies, Krajewski, Ward & Shaw, 2009). However, its geographic range is expanding both around known endemic foci and into previously free regions. It is now recognized as having a wide distribution in tropical, subtropical and temperate regions. In Europe, *A. vasorum* is established in several countries, including Belgium, Croatia, Denmark, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Netherlands, Poland, Slovakia, Spain, Sweden, Switzerland, Turkey and the United Kingdom (UK). Outside Europe, its occurrence is less defined, although known to be endemic in North America (Newfoundland, Canada), South America (Brazil and Colombia), and Africa (Uganda). Attention should be given to the fact that lack of reports of *A. vasorum* in some regions does not ensure its non-existence, thus, geographic location should not be used as the sole criterion to suspect or rule out this diagnosis (reviewed by Koch & Willesen, 2009; Traversa et al., 2010; Elsheikha et al., 2014).

Regarding prevalence, CPA varies widely between canine populations (e.g. pet, hunting or stray dogs), health status (e.g. healthy or clinically affected) and the diagnostic methods used (e.g. coprological, serological or molecular methods). A prevalence ranging from 0.3-9.8% was reported in dogs in Europe, through faecal examination (reviewed by Koch & Willesen, 2009). Using ELISA, a seroprevalence (regarded as the positivity for both *A. vasorum* circulating antigen and specific antibodies against the parasite) ranged from 0.3-1.36% in large scale

sampling of dogs from European countries (Table 2). This contrasts with the much higher prevalence detected in fox populations, ranging from 5-56%, suggesting foxes as the most important reservoir of infection (reviewed by Koch & Willesen, 2009; Morgan & Shaw, 2010). For example, in the UK, studies demonstrated a prevalence of 23.2% *A. vasorum* infection in fox populations residing in a known endemic hotspot for dog infection, compared to a countrywide prevalence of 7.3% (Morgan et al., 2008).

Table 2 - Seroprevalence of *Angiostrongylus vasorum* in dogs from European countries.

Country	Ag+ and Ab+ (%)	Ab+ only (%)	Ag+ only (%)	Sample size (n)	Reference
Hungary	1.36	2.73	1.76	1247	Schnyder, Schaper, Lukács, Hornok & Farkas, 2015a
UK	0.97	3.2	1.32	4030	Schnyder, Schaper, Bilbrough, Morgan & Deplazes, 2013a
Switzerland	0.96	2.12	1.21	6136	Lurati, Deplazes, Hegglin & Schnyder, 2015
Italy	0.8–0.9	1.5-2	0.9	265 + 447	Guardone, Schnyder, Macchioni, Deplazes & Magi, 2013
Poland	0.51	1.29	0.78	3345	Schnyder et al., 2013b
Sweden	0.39	1.48	0.70	3309	Grandi, Osterman-Lind, Schaper, Forshell & Schnyder, 2016
Germany	0.3	2.25	0.5	4003	Schnyder et al., 2013a

Factors underlying this apparent expansion are numerous and include augmented mobility of pet dogs, increased densities of reservoirs and intermediate hosts, urbanization and climate changes. Another equally plausible hypothesis includes better awareness and surveillance of *A. vasorum* among clinicians and enhanced and more readily available diagnostic tests. The spread of *A. vasorum* is probably due to a combination of these and possibly other factors, what makes this infection, unpredictable (reviewed by Morgan et al., 2005).

Information regarding CPA infection in Portugal is almost absent, with only two cases reported in dogs (Pötz, 2006; Madeira de Carvalho et al., 2009). In foxes, *A. vasorum* was first identified during the necropsy of one out of 306 (0.33%) red foxes (*V. vulpes silacea*) sampled between 1970-1987, mostly from the coastal central and southern regions of the country (Carvalho-Varela & Marcos, 1993), and by necropsy of 10 out of 62 red foxes (16.1%) collected by hunters from central Portugal (Eira et al., 2006). Later, a prevalence of 7.1% of *A. vasorum* was detected

in fresh faecal samples of red foxes collected in western-central Portugal (Figueiredo, Oliveira, Madeira de Carvalho, Fonseca & Torres, 2016).

2.2.2 Climate-matching model and seasonality

The climate is hypothesized to influence the transmission of *A. vasorum* as the activity and population dynamics of the gastropod intermediate hosts is highly affected by temperature and water availability (Morgan et al., 2009). Despite the recent spreading of *A. vasorum*, few studies have so far been conducted to predict the extent to which current or future distribution might be limited by climate. This is due to the paucity of experimental data on the effect of climate on parasite development and transmission. In fact, prediction models of the distribution of parasites and/or risks of infection generally use either detailed spatial correlation of abundance with climatic factors or extrapolate known relationships between climate and vital rates. However, both approaches require detailed and reliable data on distribution, vital rates, climatic variables and transmission parameters for parasite establishment and persistence, and these data are not available for *A. vasorum*. Furthermore, approaches based on intermediate and definitive host distribution are not appropriate for this parasite, as a wide range of mollusc species may act as intermediate hosts and as its definitive hosts are globally distributed (Guilhon & Cens, 1973; Morgan et al., 2009).

Morgan et al., (2009) performed a preliminary attempt to predict the potential spread and areas at greatest risk of parasite establishment. Climatic modelling suggests that the potential geographic range of *A. vasorum* is indeed much larger than the area currently occupied by the parasite, even without considering climate changes. Interestingly, it showed numerous regions of the world that are suitable for *A. vasorum* establishment, although not yet colonized, e.g., coastal North America, Japan, and parts of South Africa, Australia and New Zealand (Morgan et al., 2009) (Fig. 5).

Figure 5 - Predicted geographical distribution of *Angiostrongylus vasorum* (Morgan et al., 2009).

Note: Shadows indicate the eco-climatic index (ECI): black shadow represents high predicted suitability for the transmission of *A. vasorum* (ECI > 45, grey shadow intermediate values with ECI of 30 and 15, and unshaded areas ECI of zero.

Seasonal patterns have been reported, with most of the cases diagnosed from October to February in Newfoundland, Canada (Conboy, 2004), December to May in Denmark (Taubert, Pantchev, Vrhovec, Bauer & Hermosilla, 2009) and December to January in Germany (Maksimov et al., 2017). Other authors have reported the occurrence of canine *A. vasorum* outbreaks in mild and wet years, precisely when gastropods are more abundant (Cobb & Fisher, 1990; Conboy, Schenker & Strehlau, 2004). Similarly, Segovia, Torres and Miquel (2004), found a much higher prevalence of *A. vasorum* in foxes from mild and damp coastal areas of the northern Iberian Peninsula in comparison with those from the warm and dry southern interior areas. Also, hunting activity is reported to increase the exposure risk of dogs to *A. vasorum* due to a frequent contact with infected slugs in woodland areas co-inhabited by red foxes. In addition, the moderate temperatures and high rainfall that often occurs during hunting season are generally associated with a peak in terrestrial gastropod populations (Conboy, 2011).

2.2.3 Risk factors and populations at risk

Some authors consider Cavalier King Charles Spaniels (Chapman, Boag, Guitian & Boswood, 2004), Staffordshire Bull Terriers (Chapman et al., 2004), Beagles (Conboy, 2004) and hunting dog breeds (Conboy, 2004; Conboy, 2011) at higher risk of *A. vasorum* than crossbreeds. Indeed, one of the cases of CPA reported in Portugal occurred in a Cavalier King Charles Spaniel. However, other authors do not report any breed preferences (Traversa, Torbidone,

Malatesta & Guglielmini, 2008; Taubert et al., 2009; Van Doorn, Van de Sande, Nijsse, Eysker & Ploeger, 2009).

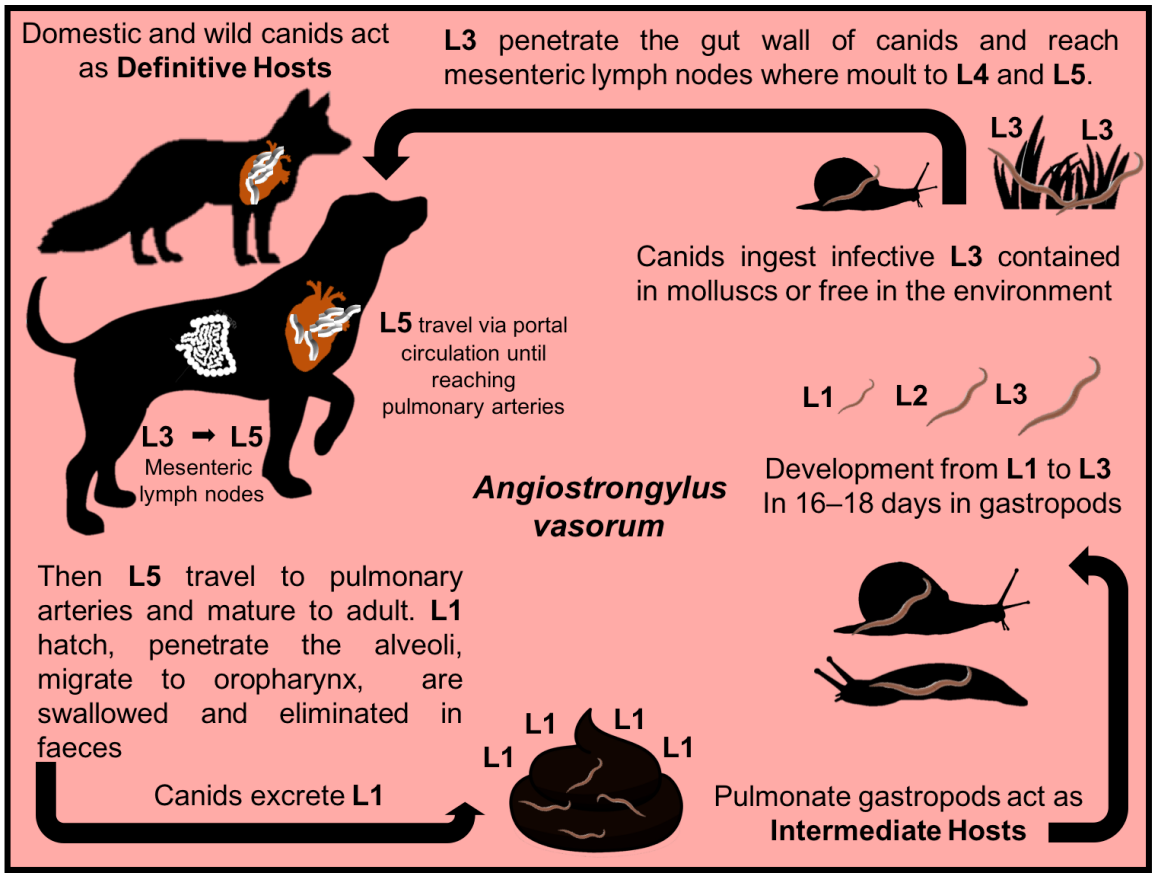
Although *A. vasorum* may affect dogs of all ages, it tends to occur more often in younger canids, with more than 50% of the affected dogs being under one year old (Boag, Lamb, Chapman, Boswood, & 2004; Chapman et al., 2004). No gender predisposition has been found in dogs (reviewed by Koch & Willesen, 2009). In foxes, no data have been found regarding age, gender or body condition and *A. vasorum* infection (Morgan et al., 2008).

2.3 Biology of *Angiostrongylus vasorum*

2.3.1 Life-cycle

The life-cycle of *A. vasorum* was described for the first time by Guilhon (1960). It is an indirect cycle with pulmonate gastropods (slugs and/or terrestrial and aquatic snails) acting as intermediate hosts. Canids acquire the infection either indirectly through the ingestion of infective L3 contained in the tissues of intermediate and paratenic hosts (by predation) or directly through chewing grass contaminated with free viable L3 (Barçante, Barçante, Dias & Lima, 2003) (Fig. 6). Then, gastropods are ingested by the definitive host and L3 released from the intermediate/paratenic hosts. After this, L3 penetrate the gut wall and migrate to the mesenteric lymph nodes, where they moult to L4 and then to L5. Then, L5 travel along the mesenteric lymphatics, hepatic and portal veins, liver and caudal vena cava, to reach the right ventricle and pulmonary arteries (as early as 10 dpi) where they mature to adults (reviewed by Rosen, Ash & Wallace, 1970). Thereafter, the worms mate and produce eggs that will develop in lung capillaries. L1 hatch, penetrate the alveolar airspace, migrate up to the oropharynx, after which they are coughed-up and swallowed and then eliminated in the faeces of infected animals. The pre-patent period is reported to be 38–57 days. However, it can range widely from 28 to 108 days (reviewed by Bolt, Monrad, Koch & Jensen, 1994). Besides, lifespan of *A. vasorum* is long and dogs have been reported to shed larvae for more than 5 years after exposure (reviewed by Rosen et al., 1970) and as many as 280.000 larvae per gram of faeces (Martin, Ashton, Simpson & Neal, 1993). Nevertheless, larvae excretion is intermittent with a great day-to-day variation in the number of larvae shed (Oliveira-Junior, Barçante, Barçante, Dias & Lima 2006).

Figure 6 - Life cycle of *Angiostrongylus vasorum* (original).



Gastropod molluscs are exposed to L1 through feeding on the faeces of infected canids. Infective L3 develop within 16–18 days in gastropods at 20–23ºC (Guilhon & Cens, 1973), although larvae development in the gastropod is assumed to be temperature-dependent (Jenkins, Kutz, Hoberg & Polley, 2006).

Several investigations had shown that most species of gastropods, including slugs (*Arion hortensis*, *Deroceras reticulatum*, *Limax flavus*, *Laevicaulus altes*); terrestrial snails (*Achatina fulica*, *Arianta arbustorum*, *Bradybaena similaris*, *Cepaea nemoralis*, *Cochlodina laminata*, *Helix pomatia*, *Helix aspersa*, *Prosopeas javanicum*, *Subulina octona*, *Succinea putris*, *Theba pisana*); and aquatic snails (*Biomphalaria glabrata*, *Biomphalaria pfeifferi*, *Physa* sp.), experimentally infected with *A. vasorum,* are suitable for the development of L3, confirming numerous potential intermediate hosts (reviewed by Rosen et al., 1970; Guilhon & Cens, 1973; Sauerlander & Eckert, 1974; Prestwood, Greene, Mahaffey & Burgess, 1981). Also in nature, the range of species found to be infected is also very broad (Ferdushy, Kapel, Webster, Al-Sabi & Gronvold, 2009), with natural infections reported in the slugs *Arion ater*, *Arion ater rufus*, *Arion lusitanicus, Arion distinctus*, *Limax maximus* and *Tandonia sowerbyi* in central Europe (Guilhon & Cens, 1973; Ferdushy et al., 2009). This wide-range of susceptible intermediate host species indicates a remarkable lack of intermediate host specificity for *A. vasorum*.

In Portugal, the *Arion rufus* and *Deroceras laeve* slugs are known to be suitable *A. vasorum* intermediate hosts, and have inclusively been described in distinct parts of the national territory (Grewal, Grewal, Tan & Adams, 2003; Bank, 2011).

2.3.2 Host range and specificity

The definitive hosts of *A. vasorum* are mainly canids, such as foxes (*V. vulpes*) (Bolt et al., 1992), wolves (*C. lupus lupus*) (Segovia, Torres, Miquel, Llaneza & Feliu, 2001), jackals (*C. aureus*) (Takacs et al., 2013), coyotes (*C. latrans*) (Bourque, Whitney & Conboy, 2005), Brazilian foxes (*Dusicyon vetulus*) (Lima, Guimaraes & Lemos, 1994) and domestic dogs (*C. familiaris*) (Guilhon & Cens, 1969).

Although not all confirmed by PCR, natural infections may also occur in non-canids, including the Eurasian badger (*Meles meles*), lynx (*Lynx canadensis*), red panda (*Ailurus fulgens*) and the river otter (*Lutra lutra*) (Conboy unpublished; Madsen, Dietz, Henriksen & Clausen, 1999; Torres, Miquel & Motje, 2001; Grondahl et al., 2005). In addition, patent experimental infections were established in the jackal (*C. aureus*), African desert fox (*Vulpes zerda*) and the Nile rat (*Arvicanthus niloticus*) (reviewed by Rosen et al., 1970). In experimentally infected cats, worms reached maturity, but the larvae were not shed in the faeces (reviewed by Bolt et al., 1994; Dias, Oliveira, Viana & Lima, 2008).

2.3.3 Role of wildlife hosts

There is still much to be understood about the dynamics of infection in wild populations and the factors underlying spill-over to dogs. The most significant wildlife reservoir in Europe for *A. vasorum* in domestic dogs is the red fox (*V. vulpes*) (Bolt et al., 1992; Morgan et al., 2008; Gerrikagoitia, Barral & Juste, 2010). In wolves, *A. vasorum* is rare, with only few cases described (Segovia et al., 2001; Eleni, De Liberato, Azam, Morgan & Traversa, 2014). Indeed, the lack of genetic diversity between the *A. vasorum* samples obtained from dogs, foxes and coyotes support the hypothesis that transmission occurs between wild and domestic canids (Jefferies, Shaw, Viney & Morgan, 2009a). It is suggested that an increase density of infected foxes in areas populated with dogs may increase the contamination of the environment with L1. This may consequently lead to a higher number of infected gastropods and potential paratenic hosts, or even to an increased number of free L3 in the environment and easily accessible to dogs.

In fact, in several countries like Canada, England or Scotland the first identification of the parasite was reported in foxes before it emerged in dogs from the same locality, suggesting the potential that infected wild canids may have in domestic dog's infection when geographic overlap occurs (Smith & Threlfall, 1973; Bourque, Conboy, Miller, Whitney & Ralhan, 2002; Morgan et al., 2008; Schnyder et al., 2013a; Helm, Gilleard, Jackson, Redman & Bell, 2009). Furthermore, recent serological investigations showed that foxes may develop a non-protective immunity of the parasite, allowing its long-term survival, therefore contributing to the establishment and dissemination of the disease (Gillis-Germitsch, Kapel, Thamsborg, Deplazes & Schnyder, 2017).

2.3.4 Morphological characteristics of *Angiostrongylus vasorum*

Adult *A. vasorum* are reddish nematodes, measuring approximately 14.0–20.5 mm in length and 0.17–0.31 mm in diameter, with a cylindrical shape that tapers at each end (Kontrimavichus & Delyamure, 1985). As members of the superfamily Metastrongyloidea, *A. vasorum* has a small buccal capsule instead of a simple oral aperture. Males are 14-18 mm in length and their tail finishes in a bursa and two spiculae, without gubernaculum (reviewed by Rosen et al., 1970; Costa, Costa & Guimaraes, 2003). Females are slightly larger (18-24 mm in length) and due to the red blood-filled intestine intertwined with the white uterus, have an appearance like the "barber's pole" females of genus *Haemonchus*. Mature females contain undifferentiated eggs in the uterus and their vulva is in the posterior region of the body, just anterior to the anus. L1 larvae are 310-400 μm in length, have an anterior cephalic button, a spine and a tail with a sinus wave curve (Guilhon, 1963; reviewed by Deplazes et al., 2016).

2.4 Pathophysiology and clinical signs of *Angiostrongylus vasorum*

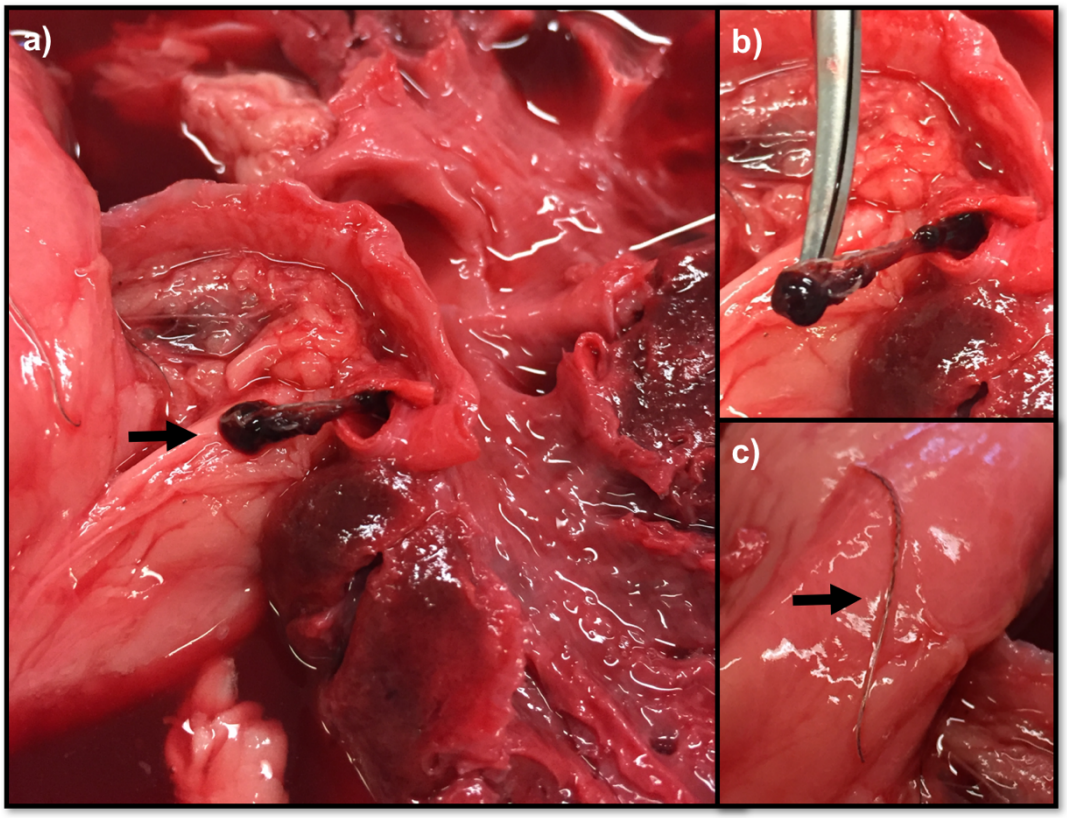
Canine CPA may be subclinical or result in a wide spectrum of clinical manifestations, ranging from minor clinical respiratory signs to severe features characterized by respiratory, cardiovascular, coagulative or neurological disorders (reviewed by Koch & Willesen, 2009). Studies show that dogs may die acutely with severe forms, be chronically ill, or be clinically normal for several months and years before showing signs of disease (reviewed by Rosen et al., 1970). The reasons for such a variable presentation are partially due to the parasitic burden, as clinical and imaging findings are severe in heavier infected dogs (Kranjc et al., 2008; Schnyder et al. 2010). Other factors may play also an important role, such as the occurrence of repetitive infections, individual host immunity and immune response (Kranjc et al., 2008). Reports of

fatal infections in which worm count was performed describe infection burdens ranging from 50–572 worms (Koch, Jensen & Monrad, 1992; Bourque, Conboy, Miller & Whitney, 2008; Oliveira-Junior, Barçante, Barçante, Ribeiro & Lima, 2004; Traversa et al., 2008). Nevertheless, as much of the pulmonary pathogenesis is triggered by the host response to L1 presence in the alveoli, the presence of mature nematodes is not mandatory to induce respiratory disease (Schnyder et al., 2009).

2.4.1 Respiratory and cardiovascular signs

Considering the parasite's primary localization in the pulmonary arteries (Fig. 7), the predominant clinical signs are respiratory, including cough (either productive or unproductive), dyspnoea, lethargy and weight loss. These signs occur due to the severe inflammatory response elicited by the presence of larvae and eggs in the pulmonary arteries and parenchyma, causing consequent verminous pneumonia, inflammation and arterial thrombosis.

Figure 7 - Adult nematodes of *Angiostrongylus vasorum* collected at the necropsy of a domestic dog (original): a) and b) *A. vasorum* nematodes involved by thrombus reaction; c) *A. vasorum* female, with the characteristic "barber's pole" aspect.



On thoracic radiography, the most common signs are alveolar infiltrate and bronchial thickening. Increased interstitial, peribronchial and alveolar patterns along with pneumothorax, subcutaneous emphysema and an abnormally wide cranial mediastinum can also be seen (Martin et al., 1993; Gallagher, Brennan, Zarelli & Mooney, 2012; Spodsberg, Miles, McEvoy & Willesen, 2013). Serpiginous or circular radiopaque areas attributed to fistulas may also happen during larvae migration into the alveoli (Capogna, Sasanelli, Lia, Spagnolo & Paradies, 2012). A correlation between time and the radiographic pattern was suggested: bronchial-interstitial pattern in an early phase (5-9 weeks post infection) and a decrease of that pattern until approximately 21 weeks when fibrosis is seen, showing that *A. vasorum* lesions cannot fully regress after anthelmintic treatment (Boag et al., 2004; Kranjc et al., 2010).

Cardiovascular abnormalities may also occur and include attenuated cardiac sounds, myocarditis, heart murmurs (grade II to VI), heart failure, periarteritis, pulmonary hypertension and right heart failure secondary to obstruction (Gould & McInnes, 1999; Chapman et al., 2004). Thoracic radiograph may show right ventricular enlargement, truncated pulmonary arteries and an increase in vertebral heart scale. Echocardiography may reveal a dilation of the right ventricle and atrium, a bulging interatrial septum, dilated vena cava, pulmonary hypertension, dilated hepatic veins and a severe tricuspid incompetence (Estèves et al., 2004; Sasanelli, Paradies, Otranto, Lia & De Caprariis, 2008).

2.4.2 Coagulopathy signs

Although coagulation disorders are less common, they are severe and more likely to be fatal (reviewed by Morgan et al., 2005). In some cases, bleeding was described as the primary and single clinical complaint in dogs infected with *A. vasorum* (Glaus et al., 2016). A variety of bleeding manifestations may be seen, such as petechial or ecchymosis, post-operative haematomas, increased post traumatic bleeding tendencies, anaemia, intracranial haemorrhage, epistaxis, haemoptysis, haematuria, haemoabdomen, gastrointestinal bleeding and melena, sub-conjunctiva and sclera haemorrhages (reviewed by Koch & Willesen, 2009; Elsheikha et al., 2014). The underlying mechanisms are still not fully understood. Indeed, coagulopathy can be wide ranging, with some animals presenting suggestive signs of primary DIC (Cury, Lima, Guimaraes & Carvalho, 2002), immune-mediated thrombocytopenia (Gould & McInnes, 1999; O'Neill, Acke, Tobin & McCarthy, 2010), secondary and tertiary coagulopathy associated with a dysregulation of the anticoagulation pathway (Cury et al., 2002; Whitley, Corzo-Menendez, Carmichael & McGarry, 2005), or even, a combination of the previous three. Nevertheless, in most patients with coagulopathy, the laboratory findings of thrombocytopenia and increased D-

dimers are consistent with low grade DIC (Adamantos, Waters & Boag, 2015). Sudden death after an acute onset of clinical disease has been described, due to cerebral haemorrhage, pulmonary artery occlusion and rupture of the femoral artery (Martin et al., 1993; Garosi et al., 2005).

2.4.3 Neurological signs

Neurological disorders are less frequent but of clinical importance. Most neurological signs occur secondary to coagulopathies (such as bleeding in or around the central nervous system), systemic dissemination of larval stages, or caused by cerebral hypoxia due to chronic cardiac insufficiency. Central nervous system signs include ataxia, amyostasia, paresis, depression, head tilt, neck pain, paralysis and seizures. As neurological defects associated with *A. vasorum* infection are difficult to diagnose using conventional radiographs, computed tomography (CT) and magnetic resonance imaging (MRI) are indicated under clinical suspicion. The presence of neurological signs in young animals with acute onset should prompt clinicians to consider testing for *A. vasorum* (Garosi, Platt, McConnell, Wray & Smith, 2005; Conboy, 2011).
Aberrant migration of adult nematodes can occur in a wide range of tissues, with reports in the eye, femoral artery, left ventricle, pericardial sac and urinary bladder (Rosenlund, Boserup & Monrad, 1993; King, Grose & Startup, 1994; Oliveira-Junior et al., 2004; Manning, 2007; Colella et al., 2016). L1 have been found at necropsy involving multiple tissues, such as adrenal gland, brain, eye, intestine, kidney, liver, pancreas, skeletal muscle, spinal cord, spleen, stomach and thyroid gland (Perry, Hertling & Kennedy, 1991; Oliveira-Junior et al., 2004; Bourque et al., 2008).

2.5 Diagnosis of canine cardiopulmonary angiostrongylosis

The definitive diagnosis of canine CPA is performed through the demonstration of the presence of adults or larval stages in the definitive hosts. Further diagnostic tests may also be performed to assess the severity of the infection, the need for supportive treatment and to provide a prognosis.

2.5.1 Parasitological diagnosis

Qualitative Baermann migration-sedimentation test is the technique of choice for the diagnosis of *A. vasorum,* allowing the recovery and the consequent identification of L1 based on its morphological features (reviewed by Koch & Willesen, 2009). *A. vasorum* L1 are 310–399 μm

in length and 14–16 μm in diameter (Zajac & Conboy, 2012), have an anterior cephalic button, and the tail ends in a sinus wave S-shaped curve (severe kink) with a dorsal spine (McGarry & Morgan, 2009) (Fig. 8). Accurate identification of the larvae must be performed to exclude other potential lungworms, intestinal parasites and free-living soil and plant nematodes that can also be present in dog faeces (Zajac & Conboy, 2012; Conboy, 2009). Important limitations of Baermann test are its limited sensitivity on a single faecal examination, the high variability in shedding of larvae, the time involved (up to 24 hours) and the fact that is too laborious to be used in large scale epidemiological studies (Verzberger-Epshtein et al., 2008). Sensitivity can be improved by pooling faeces from three consecutive days (reviewed by Koch & Willesen, 2009).

Other coprological methods such as direct faecal smear and faecal flotation to detected L1 may also be performed but with lower sensitivity (up to 54-61%) (Humm & Adamantos, 2010; Morgan & Shaw, 2010). FLOTAC, a recent improved flotation-based coprological method that allows the visualization of parasitic elements in faecal samples, showed higher sensitivity than the Baermann method for the detection of *A. vasorum* L1 (Schnyder et al., 2011a). Larvae may also be detected in bronchoalveolar lavage (Barçante et al., 2008), although with lower sensitivity and higher risk, especially in dyspnoeic patients.

Figure 8 - Tail of a first stage larvae of *Angiostrongylus vasorum* collected using Baermann migration-sedimentation test, highlighting the characteristic sinus wave s-shaped curve with a dorsal spine in the tail (arrow) (original).

2.5.2 Immunological diagnosis

Serological detection of circulating *A. vasorum* antigen (Verzberger-Epshtein et al., 2008; Schnyder, Tanner, Webster, Barutzki & Deplazes, 2011b) and parasite specific antibodies (Schucan et al., 2012) has shown to be more sensitive than faecal examination tests in dogs. Serological tools are currently considered useful techniques, either relevant to clinical diagnosis and to epidemiological surveillances studies (Schnyder et al., 2013b).

The antigen detection using monoclonal and polyclonal antibodies in a sandwich ELISA has a high specificity (94%) and sensitivity (95.7%) (Schnyder et al., 2011b). Additionally, a rapid in-clinic assay (IDEXX AngioDetect™ Test) is now available for routine detection of circulating antigens (specificity of 100% and sensitivity of 84.6%) (Schnyder, Stebler, Naucke, Lorentz & Deplazes, 2014).

An antibody detection ELISA, using *A. vasorum* adult somatic antigen purified by monoclonal antibodies has shown high sensitivity (81.0 %) and specificity (98.8 %) (Schucan et al., 2012) and has been applied along with antigen detection in several serological surveys. However, it is worth mentioning that when using antibody detection as a clinical diagnostic tool, persistence of antibodies in dogs from areas with high background levels of exposure to *A. vasorum*, could lead to false positives. Thereby, the exclusive use of antibody test alone is of limited value and should only be used to screen for exposure. The option of combining antigen and antibody detection by ELISA in clinical and epidemiological settings or combining ELISAs with coproscopic techniques is the best approach to increase sensitivity and the capacity to detect an early infection (Schnyder et al., 2013a).

2.5.3 Molecular diagnosis

As morphological studies are laborious and time consuming, molecular approaches such as the conventional PCR and the real-time PCR, have been used to identify nucleic acid sequences of *A. vasorum* and circulating DNA in definitive and intermediate hosts (Helm et al., 2009; Jefferies, Morgan, Helm, Robinson & Shaw, 2011; Patel et al., 2014; Aziz et al., 2016). Although highly specific and requiring a blood sample rather than faeces, detection of *A. vasorum* DNA offered no great advantage over the Baermann test in terms of sensitivity, and is far less sensitive than detection of circulating antigens (Jefferies et al., 2011; Schnyder, Jefferies, Schucan, Morgan, Deplazes, 2015b). PCR has also been used to detect *A. vasorum* in coprological samples (Jefferies et al., 2009b; Al-Sabi et al., 2010), although PCR inhibitors present in faeces may limit the sensitivity with no obvious advantage over the Baermann or

serological tests. PCR might be useful in the following situations: when faeces are stored for long periods with the consequent death of L1 which are not able to migrate in the Baermann apparatus; when Baermann is not suggestive of *A. vasorum* L1 given morphological alterations; or when clinical features are highly suspicious of *A. vasorum* infections but faecal analysis is not supportive. Recently, detection by quantitative PCR on bronchoalveolar lavage fluid has also been reported (Canonne et al., 2016).

2.5.4 Laboratory diagnosis

Numerous biochemical and haematological abnormalities have been noted in *A. vasorum* infected dogs, including: anaemia, increased serum β globulin fractions, eosinophilia, leukocytosis, thrombocytopenia, hypercalcaemia, increased creatinine kinase and low serum fructosamine (Cury et al., 2002; Boag, Murphy & Connolly, 2005; Willesen et al., 2006; Koch & Willesen, 2009). Nevertheless, these abnormalities greatly vary between cases, with no typical or pathognomonic consistent finding (Willesen et al., 2006; Willesen, Jensen, Kristensen & Kock, 2009; Schnyder et al., 2010). Proteinuria has also been reported in chronic infections, supporting immune-mediated renal involvement (Bourdeau, 1993).
As coagulation parameters might be inconsistent, thromboelastography is a useful technique to give additional information about the type of coagulopathy and to assess the follow-up (Cury et al., 2002; Chapman et al., 2004; Sigrist et al., 2017).

2.5.5 Imaging findings

Thoracic radiographs are probably the most valuable technique to evaluate the severity of *A. vasorum* infection, along with arterial blood gas analysis, in critical haemorrhagic patients (reviewed by Koch & Willesen, 2009). The detection of peripheral or multifocal alveolar patterns in young dogs living in endemic areas is strongly suggestive of CPA (Boag et al., 2004). The first detectable changes are the presence of a diffuse and interstitial pattern, with small focal areas of alveolar pattern, 5–7 weeks post-infection. As the disease progresses, multiple alveolar and interstitial densities can be found, mostly in caudal and peripheral areas of the lungs. In chronic cases, the pattern is more bronchial and interstitial (Mahaffey, Losonsky, Prestwood, Mahaffey & Lewis, 1981), with long-term residual findings detectable even after treatment.
High resolution computerized tomography scanning (HRCT) allows an accurate assessment of verminous pneumonia, frequently characterized by consolidation and multifocal patchy ground

glass opacities, predominantly in the peripheral parts of the caudal lungs. MRI has become a valuable modality in assessing intracranial abnormalities associated with A. *vasorum* infections, such as intraparenchymal cerebral haemorrhage and spinal cord haemorrhages (Garosi et al., 2005; Wessmann et al., 2006; Dennler et al., 2011).

Echocardiography is a useful technique to assess pulmonary hypertension. This condition is reversible and uncomplicated in mild to moderate stages, but a serious problem with a guarded prognosis in severe and chronic affected dogs. Echocardiographic findings include right atrial and ventricular dilatation, systolic septal flattening, reduced left ventricular size, dilated pulmonary trunk, tricuspid and pulmonary insufficiencies and right-sided congestive heart failure in severe cases (Nicolle et al., 2006).

2.5.6 Pathological findings

Post mortem examination and pathological analysis of *A. vasorum* infected dogs and foxes show granulomatous, interstitial pneumonia with obliterated thrombotic endarteritis, disruption of alveolar architecture, fibrosis and a thickened right ventricle (Prestwood et al., 1981; Poli, Arispici, Marconcini, Mancianti & de Monte, 1984; Bourque et al., 2008; Morgan et al., 2008). Usually, gross necropsy shows severe damage to the lungs, with firm, raised and discoloured areas ranging from pale beige to dark red with haemorrhages and enlargement of the lung lymph nodes (Schnyder et al., 2009). Histopathological analysis of the alveolar structure reveals inflammation, with neutrophils, eosinophils and multiple granulomatous foci with macrophages and giant cells and haemorrhage (reviewed by Koch & Willesen, 2009; Chapman et al., 2004). It's also frequent to observe in histological sections the presence of thrombi with larvae and eggs incorporated, as well as thickening of the arterial walls and sections of adult specimens of *A. vasorum* in arteries.

Angiostrongylus vasorum nematodes may also be detected at necropsy by careful dissection of the pulmonary artery branches and the right ventricle. Occasionally, the presence of immature and adult nematodes in ectopic locations can provide a suggestive *ante mortem* diagnosis, as in the eye, pericardium and urinary bladder (Oliveira-Junior et al., 2004; Manning, 2007). Renal involvement, including glomerulonephritis and mild granuloma formations in the kidneys has been demonstrated (Bwangamoi, 1974; reviewed by Koch & Willesen, 2009), but further investigation is needed.

2.5.7 Difficulties and misconceptions in diagnosing canine angiostrongylosis

Several points are worth to be referred regarding the diagnose of *A. vasorum*. Due to the recent spread of infection from the classic hotspots to previously uninfected areas, it is rather challenging to determine whether an animal is at a high or low risk of infection according to its location. In addition, considering the remarkable spread, it is important that veterinarians remain abreast of latest development research, to avoid the belief that *A. vasorum* is not present in the area, thus not included in the differential diagnosis (Elsheikha et al., 2014; Di Cesare et al., 2015).

Other complicating factor is the wide spectrum of clinical manifestations that could occur in angiostrongylosis, inclusively subclinical infections. This broad presentation may easily lead clinicians to overlook *A. vasorum* infection and to treat for other conditions generally considered to be more relevant and prevalent, with similar presentations (e.g., viral respiratory disease or immune-mediated thrombocytopenia). Moreover, there are still several veterinary centres that do not routinely perform the Baermann faecal examination, alleging that Baermann test requires a long time, qualified operator, fresh samples and do not always recognise the presence of *A. vasorum* in an animal (e.g., intermittent larval shedding, pre-patent infection, low larval loads or poor sample quality). Additionally, the low performance of faecal tests in terms of immediacy and sensitivity has led many clinicians to presumptively treat the animals for angiostrongylosis in suspicious cases, instead of pursuing a definitive parasitological diagnosis. Blind treatment without attempts to make a definitive diagnosis should be discouraged as it removes an important source of evidence to the clinician on local epidemiological risk, as well as important clinical information for treatment and prevention regimes. It is worth mentioning that all these difficulties in diagnosing *A. vasorum* coupled with the lack of international surveillance, led to an underestimation of the true prevalence of infection and impedes collection of data on its spread and global distribution (Elsheikha et al., 2014; Di Cesare et al., 2015).

2.6 Treatment of canine cardiopulmonary angiostrongylosis

2.6.1 Anthelmintic therapy

Treatment should be started as soon as possible, as the earlier an infected dog is treated, the less severe are the pathological changes in the lungs (Schnyder et al., 2009). A variety of anthelmintics are efficacious against *A. vasorum* (Dodd, 1973; Martin et al., 1993; Conboy et

al., 2004; Willesen, Kristensen, Jensen, Heine & Koch, 2007). Levamisole and ivermectin were routinely used in the past, but has now been replaced by safer licensed products.

Fenbendazole (Panacur™, Intervet) remains a popular treatment of choice and is efficacious in many dose regimens, ranging from 25 to 50 mg/kg, *per os*, daily for three weeks (Martin et al., 1993; Chapman et al., 2004; Estèves et al., 2004; Garosi et al., 2005; Whitley et al., 2005). Fenbendazole produces a "slow kill" of the nematodes, therefore reducing the risk of anaphylaxis. However, in many territories is still not licensed as a treatment for *A. vasorum*.

The risk of ivermectin toxicity has been reduced with the introduction of novel and safer formulations of LMs, namely selamectin, milbemycin oxime and moxidectin (Bishop et al., 2000; Novotny et al., 2000; Paul, Tranquilli & Hutchens, 2000). Presently there are two licensed, highly efficacious parasite treatments effective against adult *A. vasorum* and immature stages: one is the combination of imidacloprid 10%/moxidectin 2.5% (0.1 ml/kg) spot-on solution (Advocate®, Bayer Animal Health), that requires a single monthly spot-on application, showing a 85.2% efficacy against *A. vasorum* infection (Willesen et al., 2007); and the other is milbemycin oxime tablet, in combination with praziquantel (Milbemax®, Novartis Animal Health), that requires a weekly oral administration for four weeks to treat *A. vasorum* infection (Conboy, 2004; Conboy et al., 2004; Willesen et al., 2007; Schnyder et al., 2009).

As larval excretion may occur for up to three weeks after anthelmintic treatment, a three-day Baermann re-test is recommended earliest one month later the treatment to ensure that excretion of L1 has ceased. In addition, a new Baermann should be performed twice a year, particularly in endemic areas where re-infections are known to occur (Chapman et al., 2004).

2.6.2 Supportive therapy

Little is known about supportive treatment in *A. vasorum* infection as most of the information is retrieved from case reports or small case series. Depending on the clinical presentation, supportive treatment might be indicated. Antibiotics, bronchodilators, oxygen, fluids, blood transfusions, corticosteroids, heparin and diuretics have been reported (Chapman et al., 2004; Estèves et al., 2004).

Although dogs with life-threatening haemorrhages and coagulopathies may recover after 24-48 hours after the onset of anthelmintic treatment, transfusions of fresh frozen plasma or whole blood are advised concurrently with the anthelmintic treatment. Furthermore, hyperfibrinolysis and hypofibrinogenaemia can be treated with tranexamic acid combined with fresh frozen plasma transfusions (Sigrist et al., 2017). As complications, such as pneumothorax have been reported in the initial phases of treatment (Willesen et al., 2007), strict cage rest in the initial

2–3 days of treatment is often recommended for the most severely affected dogs (reviewed by Koch & Willesen, 2009). Oxygen administration is also indicated in dogs with respiratory compromise.

Anti-inflammatory doses of corticosteroids have been described to moderate potential anaphylactic reactions, to reduce pulmonary inflammation and to diminish secondary lung fibrosis (Manning, 2007). Immunosuppressive doses of corticosteroids are recommended in cases of immune-mediated thrombocytopenia (Soland & Bolt, 1996; Gould & McInnes, 1999). The use of adrenaline or antihistamines may also be considered to treat anaphylactic reactions. If the dogs are suffering from congestive heart failure, treatment with diuretics, angiotensin-converting-enzyme inhibitor and phosphodiesterase inhibitors are indicated to decrease pulmonary hypertension.

2.6.3 Prognosis

Prognosis for complete restitution may be influenced by the duration of infection and the worm burden. To better estimate the prognosis, animals should be classified in agreement with their clinical presentation. According to Koch and Willesen (2009), three categories might be established:

- Mild: subclinical, occasional cough, gag, no changes or mild increase in density of interstitial and bronchial tissue on radiography;
- Moderate: frequent cough, dyspnoea, moderate increase in interstitial and bronchial tissue on radiography, with possible focal alveolar pattern;
- Severe: clinical bleeding, marked dyspnoea, marked diffuse interstitial and bronchial patterns on radiography, often with localised or extensive alveolar opacities in major portions of the lung field.

Regarding the survival prognosis of canine angiostrongylosis, the literature shows mortality rates ranging from 0% in mildly to moderately affected dogs to 12.5%-13% up to 23% in dogs severely affected admitted at a referral hospital. Nevertheless, it is important to mention that in most of the cases where dogs die, the cause of death is coagulopathy (Chapman et al., 2004; Willesen et al., 2007; Koch & Willesen, 2009; Sigrist et al., 2017).

2.7 Control and prevention of *Angiostrongylus vasorum* infections

Considering the wide presence of intermediate hosts and reservoirs of infection such as wild foxes, eradication of *A. vasorum* is unfeasible. Either way, measures to avoid dogs consuming L3, either from molluscs or from the environment, might be considered. Feeding dogs exclusively indoors, cleaning regularly outdoor bowls and toys or making them less accessible to slugs/snails, are possible strategies to reduce the risk of contamination with L3 (reviewed by Elsheikha et al., 2014). In heavily contaminated areas, dog owners might be warned to avoid off-leash walking of dogs. The use of molluscicides is not advisable as many pesticides are not pet safe and may increase the availability of molluscs to dogs. Environmental control measures such as the use of nematophagous fungi have also been successfully used to destruct *A. vasorum* L1 (Braga et al., 2009; reviewed by Koch & Willesen, 2009). To prevent environmental contamination with L1 and to break the life-cycle of *A. vasorum,* proper disposal of dog faeces is recommended, particularly in public areas highly frequented by dogs. Nevertheless, data suggest that foxes are a much higher reservoir of infection when compared to domestic dogs (reviewed by Elsheikha et al., 2014).

The increasing reports of this parasite drive the need for effective anthelmintic treatment of infected dogs and even more importantly, regular prophylaxis to prevent the establishment of further infection (ESCCAP, 2010). Indeed, movement of infected and untreated dogs from endemic to non-endemic countries could present a serious threat to canine health and welfare and should be avoided. Monthly use of moxidectin and milbemycin oxime may be effectively used as *A. vasorum* prophylaxis (Schnyder et al., 2009; Böhm et al., 2014). The combination tablet of spinosad with milbemycin oxime (Trifexis®, Elanco Animal Health) was also shown to have 98.8% preventative efficacy against development of adult *A. vasorum* infection with a single treatment, and the potential to prevent the establishment of *A. vasorum* infections in dogs with monthly administrations (Böhm et al., 2014). Also, the combination of the insecticide and acaricide afoxolaner and the anthelmintic milbemycin oxime in a chewable tablet formulation (NexGard Spectra®, Merial), can prevent canine *A. vasorum* infection, with an efficacy of 94.9%, when administered at monthly intervals (Lebon et al., 2016). So far, no report has been published on resistance of *A. vasorum* to chemotherapeutic compounds.

Alternatively, dogs may be regularly checked for the presence of *A. vasorum*, by the new in-clinic serological diagnostic test or Baermann technique. As canine angiostrongylosis is increasingly being reported in regions where it was not traditionally found, veterinarians should remain abreast of latest development research and raise public awareness (reviewed by Elsheikha et al., 2014).

2.8 Public health relevance of *Angiostrongylus cantonensis* and *Angiostrongylus costaricensis*

Although *A. vasorum* has no zoonotic impact, two species of *Angiostrongylus* constitute important public health concerns: *Angiostrongylus cantonensis,* responsible for a form of eosinophilic meningitis and ocular angiostrongyliasis; and *Angiostrongylus costaricensis,* responsible for abdominal angiostrongyliasis (Centers for Disease Control and Prevention [CDC], 2015).

Angiostrongylus cantonensis, also known as the rat lungworm, was originally discovered in China (Chen, 1935). Previously confined to its traditional endemic regions of the Pacific islands and Southeast Asia, *A. cantonensis* has spread and is now considered an emerging pathogen with several reported outbreaks, and more than 2.800 cases recorded worldwide (Wang, Lai, Zhu, Chen & Lun, 2008). Definitive hosts become infected by ingesting intermediate hosts (gastropods such as snails or slugs) or paratenic hosts (e.g. frogs, crabs, prawns and monitor lizards) containing the *A. cantonensis* L3 (Wallace & Rosen, 1969; Cross & Chen, 2007; Chen et al., 2011; Yong & Eamsobhana, 2013). Definitive hosts are various species of rats, in which L3 matures until adult stage, and then mate, produce eggs, that hatch into L1 and are released in rat faeces (Prociv, Spratt & Carlisle, 2000; Cowie, 2013). Gastropod hosts ingest L1 and these go through two moults until infective L3. Gastropods are then consumed by the definitive host and the cycle repeats (Prociv et al., 2000). Humans are accidental hosts and acquire the infection by the consumption of infected raw or undercooked intermediate or paratenic hosts. Also, vegetables contaminated by L3 constitute an occasional transmission pathway (Slom et al., 2002; Tsai et al., 2004). Main symptoms include headache, stiff neck, nausea, vomiting, tingling and in more severe cases, neurologic dysfunction, coma and death (Wang et al., 2008; Wang et al., 2010). Diagnosis is primarily based on clinical signs and medical history, in which history of ingestion of intermediate or paratenic hosts of *A. cantonensis* is critical for diagnosis. Serological detection of circulating antigens or antibodies against *A. cantonensis* either in serum or cerebro-spinal fluid by ELISA provides a rapid confirmation of infection (Eamsobhana & Yong, 2009). Treatment is usually supportive with the use of analgesics and corticosteroids. Combined therapy of corticosteroids with anthelmintics (such as albendazole and mebendazole) has also been reported along with supportive treatment and surgery to remove worms from the eyes when ocular angiostrongyliasis occurs (Chotmongkol et al., 2006; Wang et al., 2008).

Angiostrongylus costaricensis is the zoonotic agent of abdominal angiostrongyliasis, an intestinal acute inflammatory condition originally described in Costa Rica (Morera & Céspedes, 1970). *A. costaricensis* has been reported in several countries of North and South America, with

nearly 200 human cases described, mostly in Central America (Graeff-Teixeira, Camillo-Coura & Lenzi, 1991; Pena, Andrade Filho & de Assis, 1995; Romero-Alegría et al., 2014). Outside America, human infections have also been reported in Africa (Zaire) and Europe (France and Spain) (Romero-Alegría et al., 2014). The definitive hosts are numerous species of rodents, with the cotton rat (*Sigmodon hispidus*) considered as the main one and the most spread (Malek, 1981). Also, dogs have been reported to act as definitive hosts for *A. costaricensis*, suggesting their potential as reservoir hosts for this parasite in urbanized areas (Rodriguez et al., 2002; Alfaro-Alarcón et al., 2015). Intermediate hosts are molluscs, mostly slugs from the family Veronicellidae (Morera & Ash, 1970). Adult parasites of *A. costaricensis* reside in the mesenteric arteries of rodents, where females lay their eggs that hatch into L1 in the intestine, and are then released in rat's faeces to the environment. To continue the life-cycle, L1 need to be ingested or actively penetrate the tissue of an intermediate host, in which they moult twice until reaching L3, the infectious stage to both humans and definitive hosts (Thiengo, 1996). Rodents are infected by eating molluscs or vegetables contaminated with L3, in which L3 may perform two vascular migratory routes: lymphatic/venous–arterial system or venous portal system (reviewed by Spratt, 2015). Humans acquire the infection via the consumption of infected molluscs or unwashed raw vegetables contaminated with gastropod's mucous containing *A. costaricensis* L3 (Ubelaker, Bullick & Caruso, 1980). Humans are accidental hosts in the life-cycle of *A. costaricensis* and this lack of host-parasite adaptation leads to the not completion of the cycle, with no elimination of larvae in the faeces. However, *A. costaricensis* triggers in humans an intense inflammatory response in the ileocecal mucosa that generates a granulomatous process with massive eosinophilic inflammatory reaction (Wu, French & Turner, 1997). Abdominal pain, fever, nausea and vomiting are the most frequent symptoms (Romero-Alegría et al., 2014). Diagnosis is difficult, in part because there are still no available reliable serological tests. History of travel to endemic areas and ingestion of raw or undercooked molluscs is critical for diagnosis, being mostly obtained via pathology (Romero-Alegría et al., 2014). No specific treatment is available for *A. costaricensis* infection as the usual anthelminthic drugs are not effective and may worsen the clinical picture (Morera & Bontempo, 1985). Most cases resolve spontaneously, although, in patients who develop severe abdominal disease, surgery is necessary to remove the affected inflamed intestinal segment.

Despite the increasing number of reports, both *A. cantonensis* and *A. costaricensis* are still underdiagnosed and underreported (Wang et al., 2008; Romero-Alegría et al., 2014). This is particularly concerning for abdominal angiostrongyliasis, a poorly understood disease of which only isolated cases are available in literature, and research is highly needed to clarify

host–parasite interaction and develop diagnostic methods and treatment (Romero-Alegría et al., 2014). Considering the social changes that have occurred in the last years, such as the increase in international mobility and the migration trend, it is important to bear in mind that the current epidemiological pattern of angiostrongyliasis is changing and that nowadays, clinical cases appear also outside their original area (Wang, Wu, Wei, Owen & Lun, 2012).

3.1 Background and research objectives

Cardiopulmonary nematodes are severe and life-threatening pet parasites that are increasingly being reported throughout Europe, constituting a major problem particularly for domestic dogs. Information about their prevalence and distribution is essential for the control of animal diseases and, in the case of *Dirofilaria* spp*.,* for the control of potentially associated illnesses in humans. However, in Portugal, accurate data on both illnesses is scarce and limited to a few studies and case reports, possibly due to general unawareness by practitioners and to low accuracy regarding the available diagnosis methods for *A. vasorum*. Additionally, possible cross reactions between *A. vasorum* and *D. immitis* were reported with the *Dirofilaria* antigen test, possibly leading to incorrect or misfit diagnoses. Moreover, the increased pet travelling and environmental changes, are leading to a spread of dirofilariosis and angiostrongylosis, being introduced into previously uninfected regions.

Taking into account the above-mentioned factors, an exhaustive multidisciplinary approach was used to characterize and assess numerous issues related to the situation of both diseases in Portugal. Among these issues, it has been considered to evaluate the occurrence and distribution of *Dirofilaria* spp. and *A. vasorum* in domestic dogs, as well as in wild carnivores, a potential infection reservoir. Secondly, the bacterium *W. pipientis* was searched in Portuguese canine populations infected with *D. immitis*. Additionally, a degree-day model based on daily temperatures registered in Portuguese meteorological stations was considered to try to assess the transmission risk period of *Dirofilaria* spp. in Portugal. Given the virulence of *D. immitis*, a new approach was attempted for mechanical extraction of *D. immitis* adult worms from the heart and pulmonary arteries. Lastly, a questionnaire was conducted to assess parasite control practices and preventive measures currently performed by pet owners and inquire their public knowledge about parasitic diseases. To conclude, a full revision of the epidemiological situation of cardiopulmonary dirofilariosis and angiostrongylosis in canids over the past 20 years was considered.

Thereby, the present study is divided in the following investigating items:

I. Perform an epidemiological survey of *D. immitis* and *A. vasorum* in domestic dogs to assess its prevalence and distribution in Portugal - Chapter 2

- **a.** Prevalence and seasonal variations of canine dirofilariosis in Portugal;
- **b.** Cardiopulmonary and gastrointestinal parasites in dogs – epidemiological study in shelters from continental Portugal;
- **c.** Molecular characterization of *Dirofilaria* spp. circulating in Portugal;

d. Seroprevalence of circulating *A. vasorum* antigen and parasite-specific antibodies in dogs from Portugal;

e. Seroprevalence of vector-borne pathogens in military dogs from Portugal.

II. Perform an epidemiological survey of *D. immitis* and *A. vasorum* in wild carnivores to assess its prevalence and distribution in Portugal - Chapter 3

a. Serological survey of heartworms *D. immitis* and *A. vasorum* in red foxes (*Vulpes vulpes*) from Portugal;

b. *D. immitis* in pinnipeds and new host record.

III. Search for DNA of the endosymbiont bacterium *Wolbachia* spp. in *D. immitis* infected dogs - Chapter 4

a. Detection of *Wolbachia* DNA in *Dirofilaria* infected dogs in Portugal.

IV. Ascertain the transmission risk of *Dirofilaria* spp. in Portugal using a degree-days model - Chapter 5

a. Transmission risk of *Dirofilaria* spp. in Portugal.

V. Develop a new minimally invasive surgical technique for mechanical removal of *D. immitis* - Chapter 6

a. A homemade snare: an alternative method for mechanical removal of *D. immitis* in dogs.

VI. Assess public perceptions about parasitic diseases and parasite control practices currently performed by pet owners - Chapter 7

a. Parasite control practices and public perception of parasitic diseases: a survey of dog and cat owners.

VII. Perform a full revision of the epidemiological situation of cardiopulmonary dirofilariosis and angiostrongylosis in canids in Portugal over the last two decades - Chapter 8

a. *Dirofilaria immitis* and *Angiostrongylus vasorum*: the current situation of two major canid heartworms in Portugal.

This thesis will present the results of the research developed at the Laboratory of Parasitology and Parasitic Diseases, at the Centre for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon, under the supervision of Professor Luís Madeira de Carvalho and co-supervision of Professor Silvana Belo and Professor Peter Deplazes. This work was complemented by annual internship periods at the Institute of Parasitology, Vetsuisse Faculty, University of Zurich, Switzerland, under the supervision of Professor Peter Deplazes. Overall, this research reflects the partnership established with several national and international institutions, namely:

- Animal and Veterinary Research Centre (CECAV), University of Trás-os-Montes e Alto Douro (UTAD), Vila Real, Portugal;
- Bayer Animal Health GmbH, Leverkusen, Germany;
- Cardiology Unit, Hospital de Santa Marta, Centro Hospitalar de Lisboa Central (CHLC), Portugal;
- Centre for Vectors and Infectious Diseases Research, National Institute of Health Ricardo Jorge, Águas de Moura, Portugal.
- Department of Veterinary Sciences, School of Agrarian and Veterinary Sciences, (UTAD), Portugal;
- Dipartimento di Medicina Veterinaria, Università degli Studi di Bari, Italy;
- Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Portugal;
- Mundo Aquático S.A. Zoomarine, Albufeira, Portugal;
- National Institute for Agrarian and Veterinary Research (INIAV, I.P.), Oeiras, Portugal;
- Portuguese Air Force, Portugal;
- Small Animal Teaching Hospital, Faculty of Veterinary Medicine, University of Lisbon, Portugal;
- Unit of Parasitology and Parasitic Diseases, University of Naples Federico II, Italy;
- Victor Caeiro Laboratory of Parasitology, Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), University of Évora, Portugal.

Overall, the results here presented include 12 scientific publications, of which 9 international articles indexed in Science Citation Index (SCI) and 3 published conference abstracts:

1. **Alho, A.M.**, Landum, M., Ferreira, C., Meireles, J., Gonçalves, L., Madeira de Carvalho, L. & Belo, S. (2014). Prevalence and seasonal variations of canine dirofilariosis in Portugal. *Veterinary Parasitology*, 206(1-2), 99–105.

2. **Alho, A.M.,** Félix, L.B., Meireles, J., Belo, S. & Madeira de Carvalho, L. (2014). Cardiopulmonary and gastrointestinal parasites in dogs – epidemiological study in shelters from continental Portugal. *XVII Congresso Sociedade Portuguesa de Parasitologia*, Faculdade de Farmácia, 20-21 Nov 2014, Coimbra, Portugal. *Acta Parasitológica Portuguesa*, 20(1/2), 122-123, C0-06. [abstract]

3. Ferreira, C., Afonso, A., Calado, M., Maurício, I., **Alho, A.M.**, Meireles, J., Madeira de Carvalho, L. & Belo, S. (2017). Molecular characterization of *Dirofilaria* spp. circulating in Portugal. *Parasites & Vectors*, 10:250.

4. **Alho, A.M.**, Schnyder, M., Schaper, R., Meireles, J., Belo, S., Deplazes, P. & Madeira de Carvalho, L. (2016). Seroprevalence of circulating *Angiostrongylus vasorum* antigen and parasite-specific antibodies in dogs from Portugal. *Parasitology Research,* 115(7), 2567-2572.

5. **Alho, A.M.**, Pita, J., Amaro, A., Amaro, F., Schnyder, M., Grimm, F., Custódio, A.C., Cardoso. L., Deplazes, P. & Madeira de Carvalho, L. (2016). Seroprevalence of vector-borne pathogens and molecular detection of *Borrelia afzelii* in military dogs from Portugal. *Parasites & Vectors,* 9, 225.

6. **Alho, A.M.**, Cardoso. L., Schnyder, M., Cortes. H., Lopes, A.P., Vila-Viçosa, M.J., Deplazes, P., Belo, S. & Madeira de Carvalho, L. (2017). Serological survey of the heartworms *Dirofilaria immitis* and *Angiostrongylus vasorum* in red foxes (*Vulpes vulpes*) from Portugal. [abstract] (*to be submitted*).

7. **Alho, A.M.**, Marcelino, I., Colella, V., Flanagan, C., Silva, N., Correia, J.J., Latrofa, M.S., Otranto, D. & Madeira de Carvalho, L. (2017). *Dirofilaria immitis* in pinnipeds and new host record. *Parasites & Vectors*, 10, 142.

8. Landum, M., Ferreira, C.C., Calado, M., **Alho, A.M.**, Maurício, I.L., Meireles, J.S., Madeira de Carvalho, L., Cunha, C. & Belo, S. (2014). Detection of *Wolbachia* in *Dirofilaria* infected dogs in Portugal. *Veterinary Parasitology,* 204, 407-410.

9. **Alho, A.M.**, Nunes, T., Rinaldi, L., Meireles, J., Belo, S., Deplazes, P. & Madeira de Carvalho, L. (2014). Transmission risk of dirofilariosis in Portugal. Proceedings of the *1st Conference on Neglected Vectors and Vector-Borne Diseases*, Cluj-Napoca, Romania, 8-11 April 2014. *Parasites & Vectors*, 7 (Suppl 1): O16. [abstract]

10. **Alho, A.M.**, Fiarresga, A., Landum, M., Lima, C., Gamboa, O., Meireles, J., Sales Luís, J. & Madeira de Carvalho, L. (2016). A homemade snare: an alternative method for mechanical removal of *Dirofilaria immitis* in dogs. *Veterinary Medicine International,* 2016, 5780408.

11. Matos, M., **Alho, A.M.**, Owen, S.P., Nunes, T. & Madeira de Carvalho, L. (2015). Parasite control practices in companion animals: a survey of dog and cat owners. *Preventive Veterinary Medicine*, 122(1-2):174-180.

12. **Alho, A.M.**, Meireles. J., Schnyder, M., Cardoso, L., Belo, S., Deplazes, P. & Madeira de Carvalho, L. (2017). *Dirofilaria immitis* and *Angiostrongylus vasorum*: the current situation of two major canid heartworms in Portugal. *Veterinary Parasitology*, *(submitted)*.

CHAPTER 2

Epidemiological survey of *Dirofilaria immitis* and *Angiostrongylus vasorum* in domestic dogs in Portugal

Chapter 2, Scientific publication 1

Prevalence and seasonal variations of canine dirofilariosis in Portugal

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* The author jointly conceived the experimental design, performed the experiments, the data analysis process and co-produced the manuscript.

Abstract

Dirofilariosis is a severe vector-borne emergent disease that is spreading worldwide and becoming a serious threat to human and veterinary public health. Portugal, a Mediterranean country, has favorable climate conditions for mosquito development and survival. At present, accurate data on the prevalence and epidemiological pattern of dirofilariosis in Portugal is scarce and outdated. To study these trends, a project was developed to assess the current prevalence of *Dirofilaria immitis* infection in shelter dogs as well as the prevalence of *Dirofilaria* species present in three coastal regions in central Portugal: Coimbra, Santarém, and Setúbal. Blood samples were collected from 696 shelter dogs during three consecutive years: 2011, 2012, and 2013. A rapid immunomigration technique was performed to detect female *D. immitis* antigens. Concurrently, to detect and identify circulating microfilariae, a modified Knott's technique and acid phosphatase histochemical staining were also performed. Of the 696 dogs sampled, 105 were positive for *D. immitis*, with an overall prevalence of 15.1%. Forty of the 105 dogs were antigen negative but were positive for *D. immitis* microfilariae. Three animals were co-infected with *D. immitis* and *Acanthocheilonema dracunculoides*, and there was also one dog infected only with *A. dracunculoides*, all confirmed by polymerase chain reaction. The highest prevalence of canine heartworm during the 3-y period was in Setúbal (24.8%), followed by Coimbra (13.8%), and Santarém (13.2%), with significant inter-district differences. Our results demonstrate a higher prevalence of dirofilariosis compared with findings of previous studies and show an increasing rate of infection in the southern areas of Portugal attributed, at least in part, to bioclimatic and ecological factors. The present study updates the epidemiological situation and correlates the risk of dirofilariosis transmission within each region. These findings are highly relevant to both human and veterinary public health, contributing to the general awareness of pet owners and veterinarian practitioners and reinforcing the need for effective control measures against vectors and preventive therapy in companion animals.

Keywords: *Dirofilaria immitis*, Dog, Heartworm, Prevalence, Portugal, Transmission dynamics.

1. Introduction

The growing incidence of human and animal dirofilariosis in endemic Mediterranean countries as well as the emergence of autochthonous cases in central and northern European regions (traditionally considered as non-endemic) provide clear evidence that this zoonotic disease is becoming a serious threat to human and veterinary public health. Several factors may account for this tragic epidemiological scenario, namely global warming, colonization by new competent vectors species, pet traveling, and human environmental interactions (Colwell et al., 2011; Genchi et al., 2011; Morchón et al., 2012; Simón et al., 2012).

Canine dirofilarial infections are vector-borne diseases caused by nematode species, specifically *Dirofilaria immitis* and *Dirofilaria repens*. Adult *D. immitis* reside in the pulmonary arteries and right heart chambers, inducing lung, vascular, and heart damage that may culminate in heart failure. Adult *D. repens* inhabit subcutaneous tissues, inducing dermatitis and subcutaneous nodules (McCall et al., 2008). In urban and rural areas, dogs are the main reservoirs for *D. immitis*, whereas cats may serve as major reservoirs for *D. repens* (Tarello, 2002). Moreover, many wild animals can also act as reservoirs for *Dirofilaria* spp., playing an important role in the perpetuation of this parasite to companion animals as a consequence of their peridomestic interactions. In Portugal, the prevalence of *D. immitis* in red foxes ranges from 3.23% in north central locations, such as Coimbra (Eira et al., 2006) to 11.8% in south central districts, such as Santarém and Setúbal (Carvalho-Varela and Marcos, 1993; Carvalho-Varela et al., 1993). Additionally, *D. immitis* also has been reported in three European otters *Lutra lutra* in Portuguese natural freshwater ecosystems (Torres et al., 2004; Saraiva et al., 2012).

Humans are accidental hosts, and the resulting pathological lesions, such as ocular, subcutaneous and pulmonary disorders, are usually asymptomatic. For this reason, infections may go undiagnosed, resulting in underestimated prevalence (Simón et al., 2012).

D. immitis and *D. repens* are transmitted by common Culicidae mosquitoes of the genera *Aedes*, *Culex*, and *Anopheles* carrying the L3 infective larval stage. In Portugal, only *Culex theileri* was found to be naturally infected with *Dirofilaria* spp. larvae. The low rate of filarial infection in this species (4.76%) (Ribeiro et al., 1983) suggests the involvement of other Culicidae mosquitoes in dirofilariosis transmission.

Despite the known endemicity of *D. immitis* in mainland Portugal, accurate data on the epidemiologic incidence are scarce and outdated. Canine dirofilariosis is endemic in mainland Portugal as well as in Madeira Island. Based exclusively on microfilariae detection, without species differentiation, prevalence rates of 16.7% in Ribatejo, 16.5% in Alentejo, 12% in Algarve, and 30% in Madeira were found (Araújo, 1996). In 2011, Balreira et al. reported a

2.1% seroprevalence of canine *Dirofilaria* infection in northern and north central Portugal, with higher rates in Aveiro (6.8%) and Coimbra (8.8%).

Recent data from Cardoso et al. (2012) using a commercial ELISA kit (SNAP® 4Dx®, IDEXX Laboratories, Inc., Westbrook, Maine, USA) reported the national average prevalence of *D. immitis* in apparently healthy dogs to be 3.6% and 8.9% in dogs suspected to have clinical signs associated with canine vector-borne disease.

Other concomitant dog filarial infections may occur in Portugal, namely *Acanthocheilonema reconditum* and *A. dracunculoides* (Gomes & Grácio, 2003). Since their pathogenic potential and public health profile is less significant than that of *D. immitis*, the correct identification of their microfilariae is critical to permit initiation of a differentiated therapeutic approach (Pantchev et al., 2011).

There are no published data on seasonal dirofilariosis transmission trends in Portugal. Therefore, an update on the epidemiological pattern and transmission dynamics is required to clearly identify which *Dirofilaria* species are currently present in the country, aiming to determine the best treatment schedule according to the species and seasonal distribution. The present paper reports the regional *Dirofilaria* findings in canine populations in different ecological areas during three consecutive years in mainland Portugal.

2. Materials and methods

2.1. Survey areas

Three estuarine areas of known endemicity for canine dirofilariosis and high-density mosquito population in central Portugal were evaluated: Coimbra (north central region, 40º12′47″ N 8º27′7″ W), Santarém (central region, 39º14′24″ N 8º40′59″ W) and Setúbal (south central region, 38º31′19″ N 8º53′9″ W). The same nine animal shelters were tested each year (October/November 2011, April/May 2012, May/June 2013) to determine trends in the prevalence of *Dirofilaria* spp. in these regions.

2.2. Animal sampling

The number of animals required was estimated using WinEpi program (stratified sample approach), taking into account the global prevalence of canine dirofilariosis in each district as well as the average number of animals in the shelters.

A total of 696 shelter dogs, including 307 males (44.1%) and 389 females (55.9%), were randomly selected (Table 1). Dogs younger than 6 months were excluded due to the long life cycle of *D. immitis*. Only dogs admitted to the kennels in the previous 6 months were included in the survey to preclude evaluating the same animal in subsequent years.

2.3. Data records

A complete record was kept for each sampled dog, including the animal's age, gender, breed, and hair coat length (short or long) as well as a photograph to confirm animal's identity. All animals sampled were strays; thus, there was no information available regarding previous heartworm preventive treatments. Additionally, deworming or heartworm preventive treatments were not routinely implemented in the surveyed shelters.

Table 1 - Detection of *Dirofilaria immitis* in shelter dogs in regions of Portugal by serological and direct techniques.

Year	Districts	No. of dogs evaluated	No. of dogs positive for *D. immitis* by any test	Antigen+ Microfilaria+	Antigen+ Microfilaria-	Antigen– Microfilaria+	PCR
2011	Setúbal	40	10 (25.0%)	4	2	4	4 *D. immitis*
	Santarém	169	25 (14.8%)	14	3	8	7 *D. immitis*
	Coimbra	99	12 (12.1%)	6	4	2	1 *D. immitis* + *A. dracunculoides*
	Total	308	47 (15.3%)	24	9	14	
2012	Setúbal	34	6 (17.7%)	4	0	2	2 *D. immitis* + *A. dracunculoides*
	Santarém	122	14[a] (11.5%)	6	1	8	7 *D. immitis*/ 1 *A. dracunculoides*[a]
	Coimbra	74	11 (14.9%)	5	3	3	3 *D. immitis*
	Total	230	31 (13.5%)	15	4	13	
2013	Setúbal	27	9 (33.3%)	3	2	4	4 *D. immitis*
	Santarém	80	10 (12.5%)	4	1	5	5 *D. immitis*
	Coimbra	51	8 (15.7%)	3	0	5	6 *D. immitis*
	Total	158	27 (17.1%)	10	3	14	
Overall		696	105[a] (15.1%)	49	16	41	

[a] Single infection by *A. dracunculoides* was excluded from overall counting.

2.4. Clinical examination and blood collection

Prior to the blood collection, a physical examination was performed to check for the presence of any abnormal clinical signs (i.e., heart murmurs, ascites, and other heartworm-associated signs). Five mL of whole blood was collected from each dog's cephalic vein, and 2.5 mL was stored in a collection tube containing EDTA; the remaining 2.5 mL was stored in a collection tube without anticoagulants. Serum was harvested following centrifugation of the clotted blood and stored at −20 ºC until analysis.

2.5. Direct and serological tests

The modified Knott's technique was performed to concentrate any microfilariae in the sample. Acid phosphatase histochemical staining was performed using the Leucognost SP® commercial kit (Merck & Co., Inc, Whitehouse Station, New Jersey, USA) in accordance to the manufacturer's recommendations to exhibit the characteristic acid phosphatase activity pattern (Chalifoux & Hunt, 1971; Peribáñez et al., 2001). Briefly, 2 mL of naphthol-AS-OL-

phosphoric acid and 3 level measuring spoonfuls of sodium acetate were dissolved in 60 mL of distilled water. Four drops of pararosaniline–HCl solution and five drops of nitrite solution were mixed in a small test tube and added to the main solution after 1 min. Air-dried blood smears made from fresh blood without anticoagulant were fixed with Leucognost® fixing mixture for 1 min, washed with distilled water, and incubated in the freshly prepared staining solution in the dark for 3 h. Finally, blood smears were washed with distilled water and examined under a light microscope (x200; x400).

2.6. Immunodiagnostic test

To test for the presence of *D. immitis* circulating antigens, WITNESS® *Dirofilaria* commercial kit (Synbiotics Corp., Europe) based on rapid immunomigration technology was used. All procedures were performed as recommended by the manufacturer.

Some microfilaremic samples that either tested positive for *D. immitis* or were in doubt by the modified Knott's technique and acid phosphatase staining were negative for *D. immitis* circulating antigens. For this reason and to ensure an accurate analysis, all of these samples were subjected to molecular techniques to confirm its diagnosis.

2.7. Molecular analysis

Dog blood DNA was analyzed using panfilarial primers DIDR-F1 (5′AGT GCG AAT TGC AGA CGC ATT GAG3′) and DIDR-R1 (5′AGC GGG TAA TCA CGA CTG AGT TGA3′) described by Rishniw et al., (2006) for molecular screening of canine filarial species. Genomic DNA from an adult *D. immitis*, dog blood infected with *D. repens*, and deionized water was used as positive and negative controls, respectively. To confirm the results after amplification for *A. dracunculoides*, the same polymerase chain reaction (PCR) products were purified using a commercial kit (QIAGEN, The Netherlands) and sequenced (performed by STABVIDA).

2.8. Statistical analysis

Statistical analysis performed with Statistical Package for the Social Sciences (SPSS) IBM v. 21.0 (SPSS Inc., Chicago, Illinois, USA) and EpiTools (AusVet Animal Health Services, Toowoomba, Australia) were used to estimate regional prevalence. Pearson chi-square analysis was performed with *Dirofilaria* infection (0–negative, 1–positive) against independent variables, including gender, coat length, and age groups (<5 y, 5–1 y, >10 y). Estimated prevalence was assessed using Agresti–Coull method. A p-value <0.05 was considered statistically significant.

2.9. Transmission risk

To assess the transmission risk of *Dirofilaria* in each of the three districts, a degree-day model based on *Dirofilaria* Development Units (DDUs) was used according to Fortin and Slocombe (1981) and Lok and Knight (1998). The number of potential days with temperatures compatible with the transmission of *Dirofilaria* larvae between mosquito and reservoirs was estimated from 2011 to 2013. Minimum and maximum daily temperatures registered in the three respective Portuguese meteorological stations were obtained from Instituto Português do Mar e da Atmosfera (IPMA). Preconditions for the model were a threshold temperature of 14ºC (below which *Dirofilaria* development will not proceed in mosquitoes), 130 cumulative DDUs for larvae to reach infectivity, and a maximum life expectancy of 30 days for mosquito vectors (Fortin & Slocombe, 1981; Lok & Knight, 1998).

2.10. Ethical considerations

The study was approved by the Commission on Ethic and Animal Welfare of the Faculty of Veterinary Medicine – University of Lisbon. All technical procedures were in accordance to National (DL 276/2001 and DL 314/2003) and European legislation regarding animal welfare and met the International Guiding Principles for Biomedical Research Involving Animals by the Council for the International Organizations of Medical Sciences.

3. Results

The number of dogs surveyed was 308 in 2011, 230 in 2012, and 158 in 2013. The estimated mean age of all dogs in the survey was 5.0 ± 3.1 y, with a minimum of approximately 6 month and a maximum of 16 y. Of the 696 shelter dogs tested over the 3-y period, 9.4% (65/696) were positive for *D. immitis* based on the antigen detection test (Table 1). Microfilariae were detected in blood samples of 12.9% (90/696) dogs by the modified Knott's technique. Acid phosphatase staining demonstrated microfilariae with two distinct bright red dots, representing the excretory and anal pores specific for *D. immitis*, in 12.1% of the animals (84/696) (Fig. 1). In the remaining samples, acid phosphatase activity was dubious and difficult to characterize either due to a low microfilariae burden or faint blood smear.

In 49 (46.7%) of the 105 *D. immitis*-infected dogs, antigen testing and acid phosphatase staining both produced positive results. Discordant results between the two tests were observed in 16 samples (15.2%) due to occult infections, only detectable by antigen testing. Similarly, discordant results were found in 41 samples (38.7%), with positive results detected by acid phosphatase staining, which were later confirmed by PCR analysis (Table 1). Of these 41

samples, 37 were identified as single infection with *D. immitis*, one as single infection with *A. dracunculoides*; the remaining three were *A. dracunculoides* and *D. immitis* coinfections.

Figure 1 - Microfilaria of *Dirofilaria immitis* after acid phosphatase histochemical staining: marked red dots show the acid phosphatase activity of the excretory and anal pores (200x).

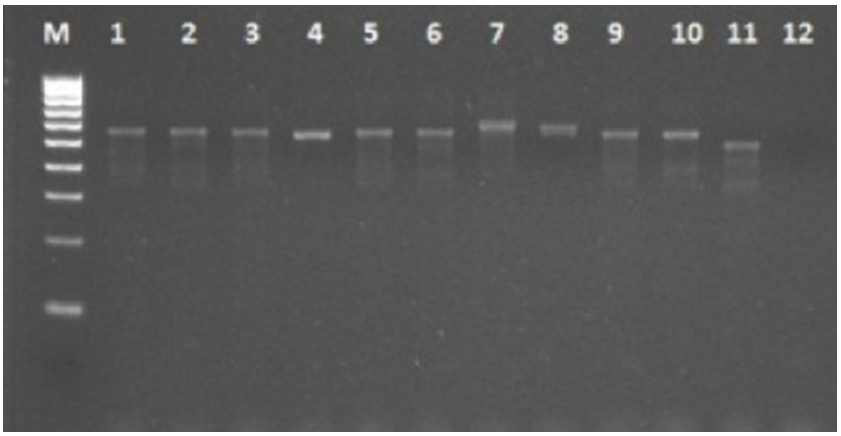
Sequenced samples (reference HG964682–HG964684, European Nucleotide Archive) showed similarities between them and 99% homology with the sequence DQ018785, specific for *A. dracunculoides* as described by Rishniw et al., (2006). No other filarids (i.e., *D. repens* or *A. reconditum*) were found during the study (Fig. 2).

Significant differences in *Dirofilaria* infection rates were observed within gender and age groups. The infection rate was higher in males (18.2% [56/307]) than in females (12.6% [49/389]) (χ^2=4.268; p=0.039). Infected animals were identified in all age groups: <5 y=11.7% (44/376); 5–10y=19.2% (48/250), and [>10 y old=18.6% (13/70)]. *Dirofilaria* infection was significantly higher in dogs ≥5 y (χ^2=7.328; p=0.026). Additionally, short-haired dogs were more frequently infected (16.7%; 65/389) than long-haired (13.0%; 40/307), although the length of dog coat was not a statistically significant factor (χ^2=1.814; p=0.178).

The overall prevalence of canine *D. immitis* infection detected over the 3-y study period in the three areas, was 15.1%. Significant interdistrict differences of *D. immitis* infection rates were observed (χ^2=8.661; p=0.013), with the highest prevalence found in Setúbal (24.8% [25/101]) [95% CI: 17.3–34.0%], followed by Coimbra (13.8% [31/224]) [95% CI: 9.9–19.0%], and Santarém (13.2% [49/371]) [CI 95%: 10.1–17.1%]. *Dirofilaria* was detected in all three districts in each of the years surveyed. The highest infection rate 33.3% was recorded in 2013, in the Setúbal area and the lowest 11.5% was recorded in 2012, in the Santarém area (Table 1;

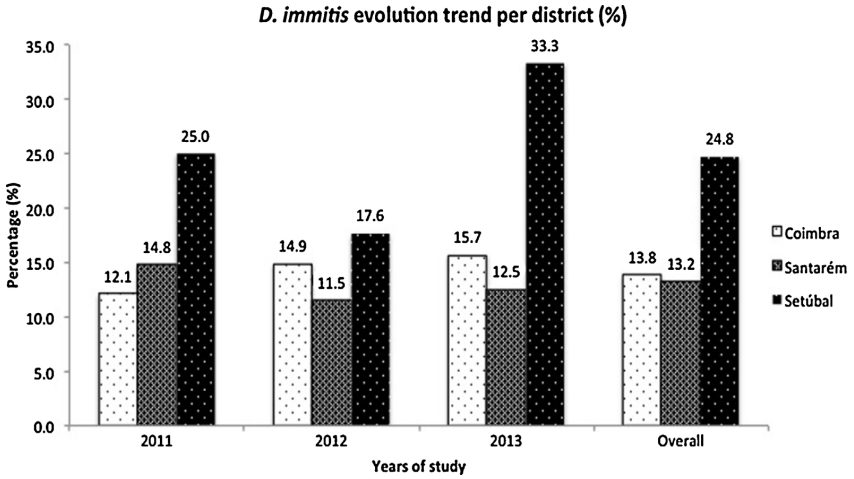
Fig. 3). Infected animals were found in eight of the nine shelters assessed (88.9%) with prevalence ranging from 5.8% to 31.9% in each shelter.

Figure 2 - Gel electrophoresis of filarial PCR products in 1.5% agarose gel.



Note: The 5.8S-ITS2-28S regions were amplified by using pan-filarial primers (DIDR-F1/DIDR-R1). The amplicons are 542-bp for *D. immitis* (lanes 1–6, 9), 584-bp for *A. dracunculoides* (lane 7), and mixed infection (*D. immitis* and *A. dracunculoides*) (lane 8); M: DNA molecular marker, 100 bp ladder; positive control from *D. immitis* (lane 10), positive control of *D. repens* (lane 11) and water for negative control (lane 12).

Figure 3 - *Dirofilaria immitis* evolution trend during 2011, 2012 and 2013 in Coimbra, Santarém and Setúbal districts of Portugal.



Considering the estimated transmission risk from 2011 to 2013, all years and all districts showed suitable conditions for *Dirofilaria* transmission. The highest average number of days with conditions for *Dirofilaria* transmission was registered in Santarém (150.7 days/y, five estimated *Dirofilaria* generations); followed by Setúbal (150.3 days/y, five estimated *Dirofilaria* generations); and Coimbra (129.7 days/y, four estimated *Dirofilaria* generations). The maximum number of potential transmission days/y (n=169.7) occurred in 2011, followed by 2013 (n=133.3) and 2012 (n=132.7). The earliest and latest period for *Dirofilaria*

transmission registered during the 3 y ranged from May 21st to November 14th in Santarém, from May 20th to November 6th in Setúbal, and from May 29th to November 2nd in Coimbra.

4. Discussion

During the 3 y of this survey conducted in Coimbra, Santarém, and Setúbal districts of Portugal, the average global prevalence of canine *D. immitis* was 15.1%. This prevalence was higher than expected when taking into account findings of previous studies conducted in central Portugal (Araújo, 1996; Balreira et al., 2011; Cardoso et al., 2012). The higher prevalence rates found may be explained, among other reasons, by the fact that this study was performed with shelter dogs, which usually are not receiving prophylactic heartworm treatments and therefore, at a higher risk of infection.

D. immitis was detected in all three districts studied from 2011 to 2013, confirming the endemicity and continuous transmission of this life-threatening disease. We suspect these findings are likely related to the geographical location of the districts (proximity to the estuarine areas of the rivers Mondego in Coimbra district, Sorraia in Santarém, and Sado in Setúbal) as well as by their mild climate. Dirofilariosis transmission depends on the presence of competent mosquito species, whose development and survival are directly related to favorable climate conditions. The higher prevalence detected in the southern region of Portugal relative to the north may be attributed, at least in part, to bioclimatic and ecological factors, since average temperatures tend to be higher in the southern areas, thus providing more favorable conditions for the vectors (Casimiro et al., 2006). Similar findings have been observed in Spain, where *D. immitis* prevalence is higher in southern areas, such as Cadiz (12%), Córdoba (18%), Badajoz (8–14%), and Alicante (13%), with the maximum rates reported in Huelva (36.7%) (Anguiano et al., 1985; Guerrero et al., 1989; MSD-AGVET, 1991; Ortega-Mora et al., 1991).

The importance of *D. immitis* in wild carnivore populations should not be overlooked, since its prevalence in red foxes is generally in agreement with infection rates in shelter dogs the present survey, with a higher rate reported in Setúbal (11.8%) and lower rate in Coimbra (3.2%) (Carvalho-Varela & Marcos, 1993; Carvalho-Varela et al., 1993; Eira et al., 2006).

A variation in the prevalence of canine dirofilariosis was observed between years in this survey, with an increase noted from 2011 (15.3%) to 2013 (17.1%), suggesting that *Dirofilaria* infection is increasing and expanding in Portugal.

The results obtained by using the forecast model show that *Dirofilaria* transmission is markedly seasonal in Portugal, with peaks in the summer, confirming data reported by Genchi et al., (2005) and Rinaldi et al., (2007). A highest transmission risk was similar for Santarém (150.7 days/y) and Setúbal (150.3 days/y), contrary to what was expected, considering the prevalence

rate for Santarém was consistently and markedly lower than that of Setúbal. Although temperatures are one influencing factor of transmission risk, according to Brown et al., (2012), many other factors promote (or interfere) with *Dirofilaria* transmission, namely precipitation, relative humidity, vegetation indices, human and animal population density, and social economic status. The abundance of rice fields in Setúbal suburban and rural areas could greatly contribute to higher larval survival and development within mosquitoes in that region. An interdisciplinary integrated approach should be considered in the future, combining all those factors as well as the interaction between animals, nematodes, and mosquitoes.

A 7-month seasonal risk period (May to November) for *Dirofilaria* transmission was also registered in the three meteorological stations. This finding may contribute to the need to implement a preventive therapy guideline in accordance with the transmission characteristics of each local area of the country, allowing a better protection of the animals during the entire risk season.

Performance of the diagnostic tests revealed higher sensitivity for direct tests (Knott's and acid phosphatase) in combination with PCR compared with serological methods. Of the 105 dogs found to be infected with *D. immitis*, 16 were microfilaria negative and antigen positive. According to Genchi et al., (2007), occult infections may be due to several factors, such as prepatency, unisex infection (by female worms), drug-induced sterility of adult filariae, or immune-mediated clearance of microfilariae.

Conversely, the 41 microfilariae-positive samples negative for antigen were confirmed by PCR. The lower sensitivity of the antigen test was unexpected, although similar findings have also been reported by Tarello (2001), who found seven dogs in Italy harboring *D. immitis* microfilariae that tested negative for *D. immitis* antigen. Furthermore, Vezzani et al., (2008) reported approximately 22% of *D. immitis* microfilaremic dogs in South America were negative for circulating antigens. The main hypotheses that justify these divergent results could be the antigen-antibody complex formation that can greatly interfere with the detection of the antigen (Brunner et al., 1988; Tonelli & Quentin, 1989; Little et al., 2014). According to Little et al. (2014), if serum is not treated with heat prior to testing, antigen–antibody complex formation can occur, trapping the antigen and inhibiting its detection. This could lead to a large number of false-negative results. Heat treatment presumably allows the disruption of antigen–antibody complexes, allowing an accurate and increased detection of *D. immitis* antigen. Other hypotheses could be a low female worm burden (between one and four) that easily decreases the sensitivity of antigen test, which has been demonstrated in several studies (Martini et al., 1996; Klotins et al., 2000; Atkins, 2003). Another theory for the failure to detect antigen in microfilaremic dogs could be related to the persistence of microfilariae following the natural or

the pharmacological death of adult worms (Vezzani et al., 2008). For this reason, the complementary use of serologic tests in combination with direct and/or molecular techniques is highly recommended to achieve an accurate diagnosis.

No other filarial species (e.g., *D. repens* or *A. reconditum*) were detected in the present survey, which was surprising considering reports of *D. repens* in other Mediterranean countries, including Spain (Cancrini et al., 2000), France (Chauve, 1997), and Italy (Tarello, 2010). At least during the period covered by the present survey, *D. immitis* appeared to be the sole etiological agent of dirofilariosis in Portugal. We expect forthcoming surveys from our project to confirm these findings, particularly in the neighboring areas of Portugal with Spain.

Reasons for the emergence/re-emergence of canine heartworm disease in Portugal are still unclear, but climate changes related to global warming are most likely implicated (Casimiro et al., 2006) as well as globalization that has led to an increased movement of pets (Colwell et al., 2011). Besides these factors, the impact of the recent socioeconomic crisis in Portugal has resulted in a reduction of the number of prophylactic treatments, particularly those directed against vectors (Madeira de Carvalho et al., 2013).

In conclusion, representative epidemiological surveys are of utmost importance not only to assess the impact of infection but also to monitor and forecast future outbreaks. The creation of a database with this and future compiled information could benefit both the veterinary and human health communities by enabling the creation of regional maps with respective associated risk and the assessment of heartworm evolution trends.

We believe that our data will be invaluable for the promotion of general awareness of pet owners and veterinarian practitioners, reinforcing effective control measures against vectors and preventive therapy in companion animals.

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Chapter 2, Scientific publication 2

Cardiopulmonary and gastrointestinal parasites in dogs – epidemiological study in shelters from continental Portugal

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Abstract

Introduction: Cardiopulmonary and gastrointestinal parasites of companion animals are worldwide distributed and currently constitute a serious public threat to Public and Animal Health. Concise data on the epidemiological situation in Portugal is scarce or merely punctual. In order to overcome the lack of available data, a large-scale epidemiological study was designed, from the North to the South of the country, to assess the parasite burden in domestic canids throughout the inland region of Portugal.

Material and Methods: Between May and September 2014, 248 blood samples and 171 faecal samples were collected from canids kept in shelters from seven districts of continental Portugal: Bragança, Guarda, Castelo Branco, Portalegre, Évora, Beja and Faro. In order to study *Dirofilaria immitis* infection, an antigen test (Witness® *Dirofilaria*) was used as well as the modified Knott technique for the identification and differentiation of microfilariae. For the observation and characterization of parasitic forms in fecal samples, qualitative methods were used namely, the fecal flotation technique, the sedimentation technique and the Baermann technique.

Results: An overall prevalence of 2.8% (7/248) for *D. immitis* infection was found. Beja registered the highest prevalence (8.9%), followed by Guarda (6.7%), Faro (2.7%) and Castelo Branco (2.5%). No positive cases of *D. immitis* were recorded in Bragança, Évora and Portalegre. Microfilariae of *Acanthocheilonema* spp. were observed in 4.5% of the samples from Beja and in 2.7% of the samples from Faro. No microfilariae of *D. repens* were found in the seven districts.

Of the total number of fecal samples analyzed, 27.5% (47/171) were parasitized, registering co-infection in 4.7% (8/171) of the samples. The most frequently parasites found were nematodes (70.2%), followed by protozoa (31.9%) and cestodes (14.9%). The gastrointestinal parasites observed with the highest prevalence were the helminths of the family *Ancylostomatidae* (12.9%), followed by the coccidia genus *Cystoisospora* (8.2%), the genus *Toxocara* spp. (5.3%), the family *Taeniidae* (4.1%) and lastly, *Toxascaris leonina* and *Trichuris vulpis* (each with 1.75%). The coinfection was reported more frequently between *Ancylostomatidae* and *Cystoisospora* (2.3%). Neither larval stages of lungworms, nor forms of *Dipylidium caninum* were found in any of the analyzed areas. Castelo Branco was the district with the highest prevalence of animals infected with gastrointestinal parasites with 36% (9/25), followed by Guarda 33.3% (5/15), Portalegre 33% (11/33), Beja 27.6% (8/29), Bragança 26.9% (7/26), Évora 25% (5/20) and Faro 8.7% (2/23).

Conclusions: These results reflect the high level of parasitism in the shelters located in the seven districts assessed, particularly for *Ancylostomatidae*, *Cystoisospora* spp., *Toxocara* spp., *Taeniidae* and *D. immitis*. Given the zoonotic potential and great impact on Public Health of many of these parasitic agents, these data highlight the urgent need to raise awareness among the general population and the veterinarian community, promoting a targeted and regular prophylaxis in pets from Portugal. Additionally, one should stress the fact that shelter environment conjugate the ideal characteristics for a continued infection particularly for parasites with a direct life cycle, some of which, with zoonotic potential. For this reason, it is also crucial to increase the awareness of those who deal regularly with animals kept in this kind of environment, as staff, volunteers and visitors, to adopt the appropriate precaution measures that may allow breaking the parasite cycle and prevent the transmission of these pathogenic agents.

Keywords: Dog, Cardiopulmonary and gastrointestinal parasites, Kennels, Zoonosis, Continental Portugal.

Chapter 2, Scientific publication 3

Molecular characterization of *Dirofilaria* spp. circulating in Portugal

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Abstract

Background: Dirofilariosis is a potentially zoonotic parasitic disease, mainly transmitted by mosquito vectors in many parts of the world. Data concerning the canine *Dirofilaria* species currently circulating in Portugal is scarce. Thereby, a large-scale study was conducted to determine the *Dirofilaria* spp. present in Portugal, based on a molecular approach, and also to optimize a reliable and highly sensitive species-specific polymerase chain reaction (PCR) assay that could be used for the simultaneous detection and differentiation of *Dirofilaria immitis*, *Dirofilaria repens*, and other concurrent filarial species in animal reservoirs.

Methods: Blood samples were collected from three districts of Portugal (Coimbra, Santarém and Setúbal) between 2011 and 2013. Samples were tested using rapid immunomigration tests (Witness® *Dirofilaria*), modified Knott's technique and acid phosphatase histochemical staining. In addition, molecular analysis was performed by amplification of the internal transcribed spacer (ITS) region using two different PCR protocols, specific for molecular screening of canine filarial species.

Results: Of the 878 dogs sampled, 8.8% (n = 77) were positive for *D. immitis* circulating antigen and 13.1% (n = 115) positive for microfilariae by the modified Knott's technique. Of the 134 samples tested by acid phosphatase histochemical staining, 100 (74.6%) were positive for *D. immitis*. Overall, 13.7% (n = 120) were positive by PCR for *D. immitis* by ITS2, of which 9.3% (67/720) were also positive by ITS1. ITS2 PCR was the most sensitive and specific method, capable of detecting mixed *D. immitis* and *A. reconditum* infections. Heterozygosity, in the form of double peaks, was detected by sequencing of both ITS regions. No *D. repens* was detected by any of the diagnostic methods.

Conclusions: The present study confirmed *D. immitis* as the dominant species of the genus *Dirofilaria* infecting Portuguese dogs, based on sequencing of ITS1 and ITS2 PCR fragments. Additionally, ITS2 PCR was the most adequate method for diagnosis and prevalence estimation.

Keywords: *Dirofilaria*, PCR, Internal transcribed spacer, Dog, Portugal.

1. Background

Dirofilariosis is a potentially zoonotic filarial parasitic disease, present in several parts of the world, transmitted mainly by mosquito vectors. The species *Dirofilaria immitis* and *Dirofilaria repens* (Filarioidea, Onchocercidae) are widely present in the Mediterranean basin and are the causative agents of cardiopulmonary and subcutaneous dirofilariosis, respectively. Both nematodes are transmitted by mosquito species of the family Culicidae and can infect domestic and wild canids and felids, causing severe pathological effects (Simón et al., 2012). *Dirofilaria immitis* is considered the most virulent filarial species in dogs, as the long-lived adult worms reside in the right ventricle and pulmonary artery, leading to pulmonary hypertension, congestive heart failure and even death (McCall, Genchi, Kramer, Guerrero & Venco, 2008; Giangaspero et al., 2013). Instead, *D. repens* adult forms live in subcutaneous tissue, where they cause dermatological problems, such as multifocal nodular and prurigo papularis dermatitis. Moreover, both species may also infect humans. *Dirofilaria immitis* pre-adult forms can cause pulmonary nodules and *D. repens* adult/pre-adult stages may induce subcutaneous and ocular lesions (Pampiglione, Canestri Trotti & Rivasi, 1995; Simón, López-Belmonte, Marcos-Atxutegi, Morchón & Martín-Pacho, 2005). Other less known canine filarial parasites, such as *Acanthocheilonema dracunculoides* (tick- and fly-transmitted) and *Acanthocheilonema reconditum* (flea- and lice-transmitted), may also infect companion animals (Lindemann, Evans & McCall, 1983; Rani, Coleman, Irwin & Traub, 2011). Adult *A. reconditum* and *A. dracunculoides* reside in the peritoneal cavity and adipose tissue of the host, and thus seem to be less virulent for canine reservoirs. Nevertheless, *A. reconditum* has also been reported in humans (Huynh, Thean & Maini, 2001).

These filarial species release circulating microfilariae (Mf) in the blood of their definitive hosts. These Mf can be diagnosed by microscopy through specific morphological identification or Mf histochemical staining (Chalifoux & Hunt, 1971; Magnis et al., 2013). Other diagnostic methods are also available, such as detection of circulating adult female antigens (currently only for *D. immitis*) and molecular approaches (Casiraghi, Bazzocchi, Mortarino, Ottina & Genchi, 2006; Venco, Genchi & Simón, 2011; Simón et al., 2012). Modified Knott's and acid phosphatase histochemical staining tests of blood smears remain the most commonly used parasitological tests for Mf detection, but are labour-intensive and require expertise. Thus, the prevalence of *Dirofilaria* spp. can be over-estimated if other filarial species are present and misidentified (Scoles & Kambhampati, 1995; Mar, Yang, Chang & Fei, 2002). Molecular protocols have been developed for reliable detection and differentiation of filarial species, in particular, a species-specific PCR assay and multiplex PCR and restriction fragment length polymorphism (RFLP) assays for simultaneous detection of different *Dirofilaria* spp., either in

the vector or in blood (Gasser, LeGoff, Petit & Bain, 1996; Watts, Courteny & Reddy, 1999; Mar et al., 2002; Nuchprayoon, Junpee, Poovorawan & Scott, 2005; Casiraghi et al., 2006; Nuchprayoon et al., 2006; Rishniw et al., 2006; Gioia et al., 2010; Latrofa et al., 2012).

Canine dirofilariosis due to *D. immitis* is known to be endemic and widely distributed in Portugal, with prevalence ranging between 0.9–27.3% in mainland regions to over 30% in Madeira Island (Araujo, 1996; Cardoso, Mendão, Madeira de Carvalho, 2012; Alho et al., 2014a; Vieira et al., 2014). *Dirofilaria repens* was recently detected for the first time, in a dog, presenting as mixed infection with *D. immitis* (Maia, Lorentz, Cardoso, Otranto & Naucke, 2016). This is a worrying finding, as the occurrence of autochthonous infections in domestic animals and the numbers of notified human cases of dirofilariosis, mainly attributed to *D. repens*, have increased substantially in several European countries in recent years (Simón et al., 2005; Pampiglione, Rivasi & Gustinelli, 2009; Genchi, Kramer & Rivasi, 2011).

The aim of the present study was to identify the *Dirofilaria* species currently circulating in Portuguese dogs through an optimised reliable and highly sensitive species-specific PCR assay for the simultaneous detection and differentiation of *D. immitis*, *D. repens* and other concurrent filariids in animal reservoirs.

2. Methods

2.1 Study areas and canine sampling examination

The study areas, as well as the clinical and parasitological procedures, were as previously described (Alho et al., 2014a). Briefly, canine surveys were conducted in kennels (run by local authorities or animal protection associations) in three districts of Portugal: Coimbra (northern-Centre region), Santarém (central-Centre region) and Setúbal (southern-Centre region) during three consecutive years: 2011, 2012 and 2013. Three surveys were carried out each year, in spring (March-April), summer (July-August) and autumn (October-November). Only dogs older than 6 months of age and residing in the kennels for at least 6 months were included.

2.2 Direct and serological tests

For clinical and parasitological examination, dogs were randomly sampled in each kennel. Physical examination was performed prior to blood collection. Blood was collected from the cephalic vein (5 ml) and stored (2.5 ml) with either anticoagulant EDTA or in a dry tube, and later processed for parasitological, serological and molecular analyses. The modified Knott's technique (KN) and the acid phosphatase histochemical staining test (AP) were used for microscopic detection and identification of Mf in blood smears. The commercial kit WITNESS® *Dirofilaria* (WT) (Synbiotics, San Diego, CA, USA) was employed for detection of *D. immitis* circulating antigen in serum.

2.3. Molecular analysis

2.3.1 DNA isolation

DNA was extracted from whole blood using CTAB (cetyltrimethyl ammonium bromide) method, adapted from Stothard, Hughes and Rollinson (1996). Briefly, 100 µl blood with EDTA (ethylenediamine tetraacetic acid) was incubated with 600 µl CTAB buffer and 0.2 mg proteinase K (Bioline, London, UK) at 56 °C for 2 h, with agitation. DNA precipitation was done with 0.6 ml absolute ethanol and the pellet hydrated in 50 µl TE buffer (10 mM Tris, 1mM EDTA, pH 7.0). DNA samples were stored at -20 °C until further use.

For *D. immitis* positive control, DNA was extracted, as above, from a small macerated section of two adult worms. For *D. repens* positive control, DNA was extracted from infected canine blood and from a worm (kindly provided, respectively, by Prof. Eva Fok, University of Veterinary Medicine, Budapest, Hungary, and by Prof. Claudio Genchi, University of Milan, Italy). Deionised water was used as a PCR negative control.

2.3.2 DNA amplification

The ribosomal internal transcribed spacer (ITS) region was amplified using two different PCR protocols for molecular screening of canine filarial species. The internal transcribed spacer 1 (ITS1) region was amplified using a semi-nested PCR as described by Nuchprayoon et al. (2003). Briefly, primers FL1-F and FL2-R were used in a first-round PCR to amplify the entire ITS region, and primers FL1-F and Di5.8S 660-R in a second-round PCR to amplify the ITS1 region, with expected amplification fragment sizes for *D. immitis*, *D. repens* and *A. reconditum* of 595, 602 and 446 bp, respectively. Amplification of the internal transcribed spacer 2 (ITS2) region was carried out using the primers DIDR-F1 and DIDR-R1 (Rishniw et al., 2006), with expected amplification product sizes of 542, 484, 578 and 584 bp for *D. immitis*, *D. repens*, *A. reconditum* and *A. dracunculoides* respectively. All PCR reactions were performed in 25 µl reaction mixtures, containing PCR buffer (Promega, Madison, WI, USA), 6 mM $MgCl_2$ (Promega), 10 pmol of each primer, 12 mM dNTPs (Promega), 2.5 U GoTaq® DNA polymerase (Promega), 10–40 ng of template DNA in deionized water. The temperature profile for both steps of the semi-nested ITS1 PCR was: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 ° C for 30 s and 72 °C for 45 s, with a final extension step at 72 °C for 10 min. Amplification of the ITS2 region had the following temperature profile: 94 °C for 2 min and 32 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, with a final extension step for 7 min at 72 °C. Amplification products were separated by electrophoresis in 2% agarose gels, stained with ethidium bromide, and visualized under UV light.

PCR analytical sensitivity was tested with serial dilutions (by a factor of 10) of DNA from a female adult worm of *D. immitis*, canine blood infected with *D. repens* and from dog blood samples with positive PCR (ITS1/ITS2) for *D. immitis*.

2.3.3 DNA sequencing and phylogenetic analysis

PCR amplicons were purified using a commercial kit (Qiagen, QIAquick PCR Purification Kit, Germantown, USA) and sequenced commercially (Macrogen, Seoul, South Korea) using the PCR primers. A BLAST search was performed to confirm species identity of the sequenced amplicons. Homologous sequences available in GenBank/EMBL/DDBJ databases were retrieved by BLAST and all sequences were aligned in BioEdit 7.2.5 (Hall, 1999). Some sequences exhibited regions of double peaks, and haplotypes were inferred manually to correspond to homozygous sequences in circulation (for ITS1), or using the programme PHASE (Stephens & Scheet, 2005) with 100 iterations, 100 thinning interval and 100 'burn-in' settings (for ITS2).

Phylogenetic relationships were estimated using MEGA 7.0 (Kumar, Stecher & Tamura, 2016), based on an alignment of regions with no gaps. The phylogenetic trees were inferred by the Maximum Parsimony method parameter, CNI (level = 1) with initial tree by random addition (10 reps) with 1,000 bootstrap replicates and a cut-off value of 74%.

2.4 Statistical analysis

Pearson's Chi-square and Fisher's exact tests were used to evaluate the differences between the proportions of species-specific infected dogs detected by each PCR protocol, among different age groups (0.5–3 years, > 3–6 years, > 6 years), gender and district as compared with parasitological and serological tests. Level of agreement was calculated using Cohen's kappa coefficient (*K*). Statistical analysis was carried out using statistical software SPSS 15.0 for Windows 10.0; a $P < 0.05$ was considered significant.

3. Results

Overall, 878 dogs (400 males and 478 females) were sampled from the three areas, Coimbra (n = 268), Santarém (n = 465) and Setúbal (n = 155). The dogs were 0.5 to 16 years old, with a median age of 4.5 years (IQR 2.5–7.0).

The analytical sensitivity of ITS1-PCR and ITS2-PCR were, respectively, 4.5 and 0.09 pg DNA from female adult *D. immitis*, 118 and 200 pg DNA from *D. repens*-infected dog blood, and 250 and 2.5 pg DNA from a *D. immitis*-infected dog. Statistical sensitivity (i.e. the proportion

of true positives) and specificity (i.e. the proportion of true negatives) of ITS2-PCR were significantly higher (McNemar test, $P < 0.05$) than of ITS1-PCR.

In the 720 dogs tested using both PCR targets, samples positive for ITS1-PCR were also positive for ITS2-PCR. Higher analytical sensitivity was observed for ITS2-PCR, with 12.9% of the blood samples positive for *D. immitis* (Table 1). Using ITS2-PCR it was possible to amplify, not only both species of *Dirofilaria* spp., but also *A. reconditum* in canine blood. Two samples that were ITS1-PCR-positive for *D. immitis*, were characterized by ITS2-PCR-RFLP as *A. reconditum* (accession number ENA: HG964682–HG964684) and were not included in the calculations. DNA from species (*D. immitis* and *Acanthocheilonema* spp.) was detected by ITS2-PCR in two samples.

Table 1 - Performance of ITS1 *vs* ITS2-PCR in 720 dog samples.

	D. immitis		***A. reconditum***	**Mixed**	***K***	***P***
	Positive (%)	**Negative (%)**	**Positive (%)**	**Positive (%)**		
ITS1	67 (9.3)	652 (90.6)	1 (0.1)	0 (0)	0.767	0.037
ITS2	93 (12.9)	620 (86.1)	5 (0.7)	2 (0.3)		

K: level of agreement ($K = 0.767$, $P = 0.037$) between each pair of tests (positive or negative results in both tests)

The performance of the PCR with the highest analytical sensitivity (ITS2) was compared with serological and direct parasitological tests for all samples (Table 2).

Table 2 - Prevalence of filarial infection according to the diagnostic assays performed.

	Total no. of samples	***D. immitis***	***Acanthocheilonema* spp.**	**Mixed**
		Positive (%)	**Positive (%)**	**Positive (%)**
Witness	878	77 (8.8)	–	–
Knott	878	115 (13.1)	–	–
Acid phosphatase	134	100 (74.6)	2 (1.5)	–
ITS2	878	120 (13.7)	5 (0.6)	2 (0.2)

Out of the 878 samples tested, *D. immitis* circulating antigen was detected in 77 (8.8%) by WT, whereas Mf were found in 115 (13.1%) stained slides by KN method. Samples with inconsistent results between WT and KN (n = 19 WT-positive, KN-negative) and KN-positive blood slides (n = 115) were submitted to AP analysis (n = 134). Out of the 134 stained slides, *D. immitis* Mf were identified in 100 (74.6%) and *A. reconditum* in two (1.5%). *Dirofilaria repens* was not identified in blood smears through any method. ITS2-PCR and KN presented the highest level of agreement (Cohen's kappa coefficient), which was lower, but also statistically significant, between ITS2-PCR and WT (Table 3).

Table 3 - Agreement between ITS2-PCR in relation to direct and serological methods.

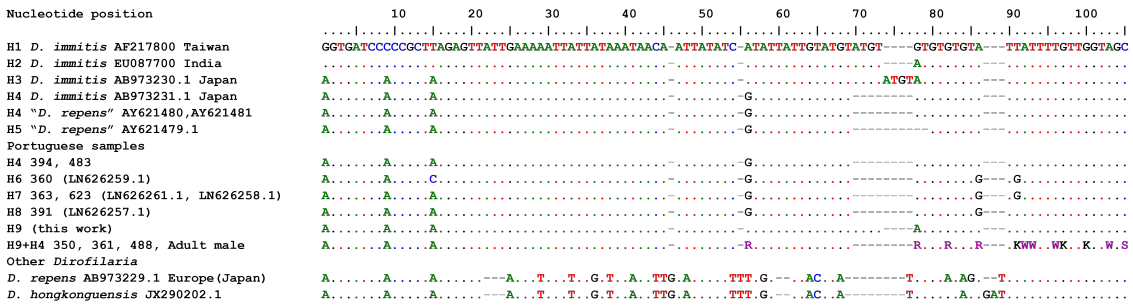
Test	Total no. of samples	Positive (%)	Negative (%)	*K*	*P*
Witness	878	65 (84.4)	739 (92.3)	0.593	0.042
Knott	878	107 (93.0)	750 (98.3)	0.930	0.018*
Acid phosphatase	134	97 (97.0)	14 (43.8)	0.513	0.088

K: level of agreement between each pair of tests (positive or negative results in both tests). *P < 0.05

3.1 Characterization of *Dirofilaria* spp.

Sequences obtained from selected ITS1 and ITS2 PCR products were analysed and deposited in GenBank under accession numbers LN626257–LN626259 and LN626261 (samples 391, 623, 360 and 363, respectively; complete ITS region); KY014643–KY014648 (samples 483, 394, 350, 361, 488, female adult worm, respectively; ITS1) and KY644132–KY644141 (samples 1, 7, 8, 29, 52, 483, 723, 732, 758, 846, respectively; ITS2). Three out of nine ITS1 sequences analysed from PCR products obtained from canine blood were found to have a string of double peaks, as did a female *D. immitis* worm (Fig. 1). The haplotypes for the ITS1 heterozygous sequences were inferred manually by assuming one sequence to be identical to the most common homozygous sequence (or haplotype) found in local samples, which in this case was H4 (Fig. 1). H4 was found in one sample from Japan, as well as the sequences AY621480.1 and AY621481.1, labelled as *D. repens* in the GenBank database (Fig. 1). The other inferred haplotype (H9) presented similarities with sequence EU087700, from India, but only from position 50 onwards in the alignment in Fig. 1. The region up to position 20 was more similar to other Portuguese and Japanese samples.

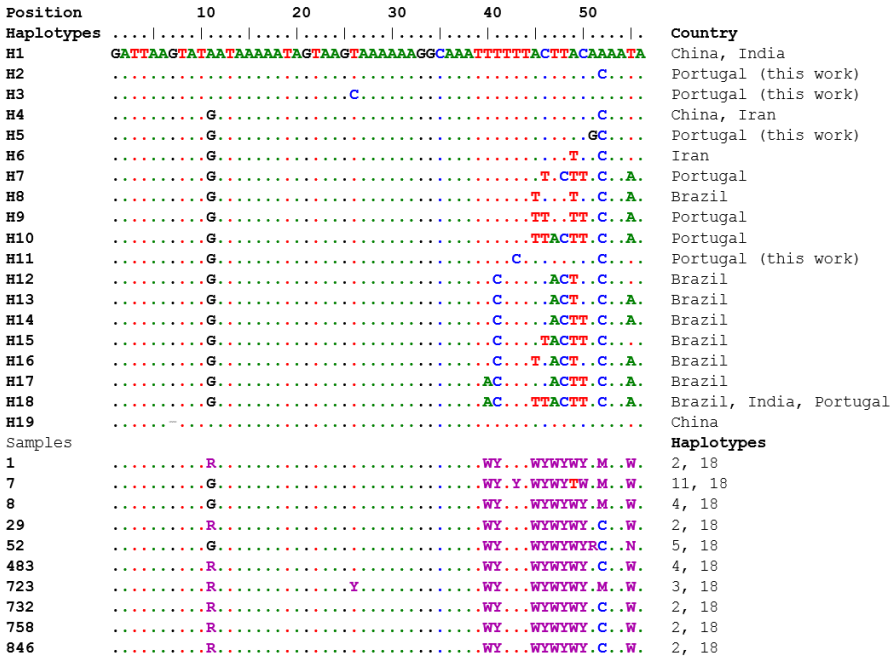
Figure 1 - Alignment of heterozygous ITS1 sequences of *D. immitis* from Portuguese canine samples.



Note: The haplotypes were inferred based on circulating haplotypes, considering the most parsimonious hypothesis that at least one haplotype is the same as the most common in circulation in the population. The first position on the alignment corresponds to position 604 of the first sequence, AF217800, reversed. The nucleotide codes K, R, S, and W, correspond, respectively to T/G, A/G, G/C and A/T.

ITS2 sequences also presented several heterozygous sites, in particular after an A and T rich region. By comparison with sequences obtained from a BLAST search, the ITS2 sequences obtained here were most similar to *D. immitis* and quite distinct from *D. repens* sequences present in GenBank, as revealed by phylogenetic analysis (not shown). Statistical reconstruction of haplotypes by comparison with sequences present in GenBank identified 19 different haplotypes (Fig. 2). All heterozygous Portuguese samples included haplotype H18, which was present in sequences from India and Brazil (dog). Five samples had H2 as the other haplotype, which has no correspondence in the database, and two samples had haplotype H4, which was present in sequences from China (red panda) and Iran (dogs). The main difference between haplotype H18 and other haplotypes was a gap of two nucleotides in a T repeat. The other three haplotypes identified (H3, H5 and H11) were not found elsewhere in the database. A BLAST analysis of the entire ITS region showed greatest similarities to *D. immitis*, with a sequence similarity that ranges from 89% to 97% with sequences available at NCBI database (JX866681.1; DQO18785.1; JX866681.1; FJ263464.1; FJ2634571; HM126606.1).

Figure 2 - Alignment of heterozygous ITS2 sequences of *D. immitis* from Portuguese canine. samples.



Note: Haplotypes 7, 9, 10 and 18 include Portuguese sequences with codes LN626262-7 that are from infected mosquitoes. Haplotypes were inferred using the programme PHASE, by comparison with homozygous sequences, with 100 iterations, 100 thinning interval and 100 burn-in settings. The first position on the alignment corresponds to position 162 of the sequence, EU087699 (H18). The nucleotide codes are as standard (M, R, Y and W, correspond, respectively to A/C, A/G, C/T and A/T). Base assignment in position 11 (R) is considered uncertain (probability of 58-9%), as is for position 26 (Y) of sample 723 (50%), position 43 (Y) of sample 7, and position 51 (R) of sample 52 (50%). Haplotypes: H1: JX481279, JX866681, EU182329; H4: U182331, JN084166, JX889634, JX8896351, JX889636, JX889637, JX889638; H6: JN084168; H7: LN626265; H8: FJ263455; H9: LN626264/66; H10: LN626262; H12: FJ263458/66/67; H13: FJ263459/60/63; H14: FJ263457/64; H15: FJ263468, H16. FJ263461; H17: FJ263465; H18: EU087699, FJ263456, LN626263.1, LN626267.1; H19: EU182330. Haplotypes H2, H3, H5 and H11 result from inference.

3.2 Pattern of canine *D. immitis* infection related to gender and age

Based on ITS2-PCR, the prevalence of *D. immitis* infection found in males (63/400; 15.8%) was significantly higher (P = 0.032) than in females (57/478; 11.9%). There were also significant differences (P = 0.01) in prevalence between age groups; the highest was found in dogs > 6 years of age (76/426; 17.8%), followed by the group with > 3–6 years of age (32/265; 12.1%) and the lowest in the 0.5–3 years age group (12/187; 6.4%). Similarly, statistically significant differences (P = 0.016) in prevalence were found between districts: Setúbal had the highest (29/155; 18.7%), followed by Santarém (63/455; 13.8%) and Coimbra (28/268; 10.4%).

4. Discussion

The application of molecular analyses targeting filarial genomic DNA in blood samples proved in this work to be a highly sensitive and specific analytical tool for the diagnosis and simultaneous characterization of canine filarial infections (Rishniw et al., 2006; Latrofa et al., 2012; Ionică et al., 2015). In comparison with serological and parasitological methods, PCR provided more reliable data for clinical and epidemiological purposes.

In the present study, the ITS2-PCR had higher analytical sensitivity and specificity than the ITS1-PCR, particularly in samples with low microfilaraemia (< 5 Mf per 20 µl of blood), for which ITS1 amplification failed or gave non-specific results. In addition, even in single or mixed infection cases, species identification of the filariae in infected dogs was also more consistent for ITS2 (Table 1).

Although parasitological and serological methods are still the most frequently used techniques for the diagnosis of canine dirofilariosis (American Heartworm Society, 2014), the present results showed that ITS2-PCR performs better in different aspects (sensitivity, specificity and species identification), thus contributing to improve diagnosis and to provide a more accurate estimation of the epidemiological pattern in the country. The ITS2-PCR assay detected mostly *D. immitis* single infections, but also 5 (0.6%) cases of *A. reconditum* and 2 (0.2%) of mixed infections (*D. immitis* + *A. reconditum*) (Table 2). ITS2-PCR was the most sensitive method, but with very similar analytical sensitivity to KN, followed by WT.

Agreement was strongest and statistically significant between PCR-ITS2 and KN test, but the molecular assay has the advantage of detecting filarial DNA in co-infected animals. Agreement between ITS2-PCR and AP or WT was much weaker. Serology is still useful for epidemiological surveys, as it can be faster and easier to use, allowing results launching to dog owners in a short time. However, detection of *D. immitis* DNA in unapparent infections can complement serology in canine surveys.

Molecular results based on ITS2-PCR also confirmed previous findings of *D. immitis* infection in dogs related to sex, age, regional distribution and prevalence (Alho et al., 2014a). In fact, previous results based on WT, KN and AP tests have also shown a higher prevalence in male dogs, older than 6 years of age and from Setúbal, confirming the North-South prevalence increase trend, as reported previously based on a fast serological diagnostic kit (Cardoso et al., 2012).

Sequence analyses of ITS1 and ITS2 fragments identified a high number of samples with at least two different alleles, which differed in sequence length, as per the inferred haplotype sequences. Although at least one of the alleles detected in each ITS region had also been found in isolates from Portugal and other regions, some samples had inferred haploid sequences that were described here for the first time. It was not possible to determine if the parasites were heterozygous or if these were cases of mixed infections in the dog. However, one adult worm presented the same heterozygous profile for ITS2, and the same ITS heterozygous patterns had been observed in the PCR product from a mosquito in Portugal, *Aedes detritus* (*s.l.*) (Ferreira et al., 2015). PCR on individually isolated Mf should clarify this issue. It is of note that some ITS1 sequences in the database had been erroneously labelled as *D. repens*, when, in fact, they correspond to *D. immitis*. Such observations raise the question over earlier publications of *D. repens* occurrence or prevalence based on this target.

Acanthocheilonema spp. are also common filarial nematodes that infect dogs in Europe and, although less virulent for animals, identification of Mf of this species in blood samples by microscopy is complex and misdiagnosis as *D. immitis* can often occur. The species-specific ITS2-PCR applied in this study detected a 0.8% prevalence of *A. reconditum*, which is similar to the prevalence found by Menn, Lorentz and Naucke (2010).

The present study showed that *D. immitis* remains, so far, the dominant species of *Dirofilaria* genus in Portugal, as confirmed by sequencing of ITS1 and ITS2 fragments from canine blood samples. These results are consistent with the results by Ferreira et al. (2015), who only detected *D. immitis* in mosquito vectors collected in the same time period in the same districts.

However, *D. repens* has recently been identified in one dog in the Algarve (Maia et al., 2016) in southern Portugal. The Algarve has the highest number of days per year with suitable conditions for *Dirofilaria* transmission (Alho et al., 2014b) and it is, thus, likely that it has been the point of introduction of this species in Portugal. Although with very low prevalence, the presence of *D. repens* in the Algarve is worrying since this species has been implicated in the increasing number of reports of human dirofilariosis in Europe (Genchi et al., 2011). Such introduction was expected, as is the establishment and an increase in prevalence of this parasite species in Portugal, given the ongoing north- and eastward expansion of both *Dirofilaria*

species that has been observed. Such expansion has been mainly attributed to global warming, as well as environmental changes, which promote the expansion of mosquito vectors, along with the increased international mobility of infected vertebrates (Genchi, Rinaldi, Mortarino, Genchi & Cringoli, 2009; Pampiglione et al., 2009; Morchón et al., 2012; Fuehrer et al., 2016). Moreover, many wild animals can also act as sylvatic reservoirs for *Dirofilaria* spp., thus maintaining transmission of this parasite. In Portugal, the prevalence of *D. immitis* in red foxes, as determined by necropsy, has ranged from 3.2% in northern-Centre locations, such as Coimbra (Eira, Vingada, Torres & Miquel, 2006), to 11.8% in southern and central-Centre districts, such as Santarém and Setúbal (Carvalho-Varela & Marcos, 1993). Additionally, in a national serological survey conducted in red foxes, 8.5% were positive for *D. immitis* circulating antigen, with positive animals found in northern and southern areas of Portugal (Alho et al., 2016). *Dirofilaria immitis* has also been reported in three Eurasian otters, *Lutra lutra*, in Portuguese natural freshwater habitats (Torres et al., 2004; Saraiva et al., 2013) and, recently, in a collection of pinnipeds from Algarve (Alho et al., 2017).

5. Conclusions

In conclusion, our data strongly suggest that *D. immitis* is the main etiological agent of dirofilariosis in Portugal and that PCR of the region ITS2, as applied here, could be a valuable tool for the diagnosis and screening of filarial infections in dogs, given its fast, accurate, specific detection and differentiation of *Dirofilaria* spp. from other concurrent blood microfilariae.

Abbreviations

AP: acid phosphatase histochemical staining test; CTAB: cetyltrimethyl ammonium bromide; EDTA: ethylenediamine tetraacetic acid; ITS: internal transcribed spacer; Mf: microfilariae; KN: modified Knott´s technique; PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism; WT: kit WITNESS® *Dirofilaria*.

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Availability of data and material

All data generated or analyzed during this study are included in the article and its Additional files. Sequences obtained from selected PCR amplicons were deposited in the GenBank database under accession numbers LN626257–LN626259 and LN626261 (samples 391, 623, 360 and 363; complete ITS region); KY014643–KY014648 (samples 483, 394, 350, 361, 488, female adult worm, respectively; ITS1); and KY644132–KY644141 (samples 1, 7, 8, 29, 52, 483, 723, 732, 758, 846, respectively; ITS2).

Authors' contributions

SB and LMC conceived and designed the research project. AMA, JM and LMC participated in the field work, conducted clinical examination and sample collection. AMA performed direct and serological analysis. CF, AA and MC carried out the PCR reactions. MC and IM carried out sequence analyses and alignments. CF, AA, AMA, IM and SB wrote the paper and supervised the statistical analysis**.** All authors contributed, read and approved the final manuscript.

Ethics approval

All the clinical procedures in this study were in accordance with Portuguese (Decree-Laws no. 314/2003 and no. 113/2013) and European legislation for the protection of animals and met the International Guiding Principles for Biomedical Research Involving Animals by the Council for the International Organizations of Medical Sciences. The protocol was approved by the Committee on Ethics of Animal and Animal Welfare (CEBEA) of the Faculdade de Medicina Veterinária, Universidade de Lisboa.

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Chapter 2, Scientific publication 4

Seroprevalence of circulating *Angiostrongylus vasorum* antigen and parasite-specific antibodies in dogs from Portugal

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Abstract

Angiostrongylus vasorum is a nematode that lives in the pulmonary arteries and right cardiac ventricle of domestic dogs and wild canids. It is increasingly being reported in several European countries and North America. This parasite induces inflammatory verminous pneumonia, causing severe respiratory disease in dogs. In some instances, coagulopathies, neurological signs and even death may occur. Scant data are available regarding the occurrence of *A. vasorum* in Portugal. Therefore, sera of 906 shelter dogs from North to South mainland Portugal were collected. ELISAs to detect *A. vasorum* circulating antigen and specific antibodies against this parasite were performed. A total of six dogs [0.66 %, 95% confidence intervals (CI) 0.24–1.43] were positive for both *A. vasorum* antigen and antibody detection, indicating an active infection, and 12 dogs (1.32 %, CI 0.68–2.30) were *A. vasorum* antibody-positive only. Regions with antigen- and antibody positive animals overlapped and were distributed over nearly all sampled areas in the country. This is the first large-scale ELISA-based serological survey for *A. vasorum* in dogs from Portugal. The endemic occurrence of *A. vasorum* in dogs from different geographical areas of Portugal is therefore confirmed.

Keywords: *Angiostrongylus vasorum*; Dog; ELISA; Seroprevalence; Epidemiology; Portugal.

1. Introduction

Angiostrongylus vasorum, also known as the French heartworm*,* is described to have apparently spread in the last decade into previously uninfected areas (Helm et al., 2010). It is a potentially lethal parasite that resides in the heart and pulmonary arteries of dogs and wild carnivores, with gastropods acting as obligate intermediate hosts (Guilhon & Cens 1973). This nematode may cause a wide spectrum of manifestations in dogs, ranging from mild (or even absent), to severe forms that can be fatal. Respiratory signs (coughing and dyspnoea), bleeding disorders (haemorrhages) and neurological signs are the most frequent clinical features described. Howbeit, non-specific signs such as depression, weight loss, anorexia and exercise intolerance may also be present (Chapman et al., 2004; Wessmann et al., 2006; Koch & Willesen 2009). Such a wide variety of clinical signs makes it challenging to confirm or exclude a diagnosis of canine angiostrongylosis based exclusively on a clinical assessment.

A definite diagnosis can be reached using the Baermann method, through the detection of *A. vasorum* first stage larvae (L1), with the characteristic kinked tail, dorsal spine and notch feature (Guilhon & Cens, 1973). FLOTAC, an improved flotation-based coproscopic method, also allows for the visualization of *A. vasorum* L1 in faecal samples, with a good sensitivity (Schnyder et al., 2011a). However, due to prepatency, intermittent larval excretion and the possible occurrence of mixed lungworm infections, copromicroscopic techniques have limitations concerning sensitivity and specificity. Besides, by the time dogs start to be positive in Baermann or FLOTAC, damage to the lung parenchyma is already present and recovery is more difficult (Guilhon & Cens 1969; Neff, 1971; Dennler et al., 2011). Newly developed diagnostic techniques, such as PCR (Jefferies et al., 2009; Al-Sabi et al., 2010) and serological methods (Schnyder et al., 2011b; Schucan et al., 2012) have been developed to detect infected animals. Serological methods were shown to be highly suitable for both individual and massive screening of dog populations. In fact, *A. vasorum* serologies require single serum samples instead of repeated faecal samples and allows for rapid detection of infection*,* shortly before or contemporaneously with patency (Schnyder et al., 2015b).

Regarding the geographical distribution of *A. vasorum*, southern France (Guilhon & Cens 1969; Bourdeau, 1993), south-east England and Wales (Jacobs & Prole 1975; Simpson & Neal 1982) and Denmark (Bolt et al., 1992) were traditionally considered areas with high endemic foci, while sporadic cases were diagnosed all over Europe. Nowadays, *A. vasorum* has a very heterogeneous distribution with reports suggesting the presence of endemic hotspots in many areas, namely in Croatia (Rajkovic-Janje et al., 2002), Italy (Della Santa et al., 2002; Guardone et al., 2013), Switzerland (Staebler et al., 2005), Germany (Staebler et al., 2005; Barutzki &

Schaper 2009), Spain (Segovia et al., 2004; Mañas et al., 2005), Greece (Papazahariadou et al., 2007), Poland (Demiaszkiewicz et al., 2014), Slovakia (Miterpakova et al., 2014), Hungary (Schnyder et al., 2015a) and others. Several hypotheses have been raised to explain this possible expansion, such as increased movements of pet dogs and increased fox populations even in urban areas, suggesting that new areas are open to colonisation (Morgan et al., 2009).

In Portugal, knowledge concerning the current situation of *A. vasorum* infection in domestic and wild canids is poor. No studies conducted so far showed positive results and no surveillance mechanisms are in place to assess its prevalence or geographical range. *A. vasorum* was first identified during the necropsy of one of 306 red foxes (*Vulpes vulpes silacea*) collected between 1970-1987, in the coastal central and southern regions of Portugal, (Carvalho-Varela & Marcos, 1993) and more recently, in the littoral centre of Portugal, with a prevalence of 16.1% (Eira et al., 2006). Excluding foxes, *A. vasorum* was sporadically identified in domestic dogs, with three positive cases diagnosed in the last few years in the Lisbon area (Madeira de Carvalho et al., 2009, 2013; Nabais et al., 2014). A serological study using a commercial *A. vasorum* antigen kit (Angio Detect^{TM} Test, IDEXX Laboratories) tested negative on the 120 surveyed dogs from the Algarve region (Maia et al., 2015).

The present serological study aimed to increase the knowledge about the occurrence and geographical dispersion of *A. vasorum* infections in Portugal.

2. Material and methods

A total of 906 shelter dogs randomly distributed from north to south of mainland Portugal were studied. All animals were stray dogs and no information was available regarding previous preventive treatments. Blood samples (2–3 ml) were collected from the cephalic vein and serum was separated by centrifugation and stored at −20°C until use. Sera were tested at the Institute of Parasitology, Vetsuisse Faculty, University of Zurich, Switzerland, for the presence of circulating *A. vasorum* antigens using monoclonal and polyclonal antibodies in a sandwich ELISA, with a sensitivity of 95.7% and a specificity of 94.0%, as previously described (Schnyder et al., 2011b). Additionally, a sandwich ELISA (sensitivity 81.0%, specificity 98.8%) using *A. vasorum* adult somatic antigen purified by monoclonal antibodies (mAb Av 5/5) was used for specific antibody detection (Schucan et al., 2012). Test thresholds (Schnyder et al., 2013a) were regionally determined with 300 randomly selected samples based on the mean value of optical density (A_{405} nm) plus 3 standard deviations. All test runs included a background control, a conjugate control, three positive control sera from three experimentally infected dogs and two negative control sera from uninfected dogs.

The collected data were analysed using a geographical information system (GIS) program

(RegioGraph 10, GfK GeoMarketing, Bruchsal, Germany) to visualize the regional distribution of collected and analysed serum samples and *A. vasorum* antigen- and/or antibody-positive samples. The locations of positive samples were displayed on maps with administrative and postcode boundaries based on the Portuguese three-digit postcode as points of reference. Excel 2007 for Windows (Microsoft Corporation, Redmond, USA) was used to calculate the prevalence values and their 95% confidence interval (CI).

3. Results

A total of 0.66% of the dogs (n=6, 95% CI: 0.24-1.43) were positive for both *A. vasorum* antigen and antibody detection and 12 dogs (1.32%, 95% CI: 0.68-2.30) were *A. vasorum* antibody-positive only. Additionally, a total of 1.99% (n=18, 95% CI: 1.18-3.12) of the dogs were *A. vasorum* antigen-positive only (Table 1). Regions with antigen- and antibody-positive animals overlapped and were distributed over nearly all sampled areas in the country (Fig. 1).

Figure 1 - Occurrence of *Angiostrongylus vasorum* detected by ELISA in 906 dogs from Portugal: dark grey areas represent the origin of the tested dog sera; dogs positive for antigen and antibody are represented by red dots; dogs positive only for antibody are represented by green squares; dogs positive only for antigen are represented by blue triangles.

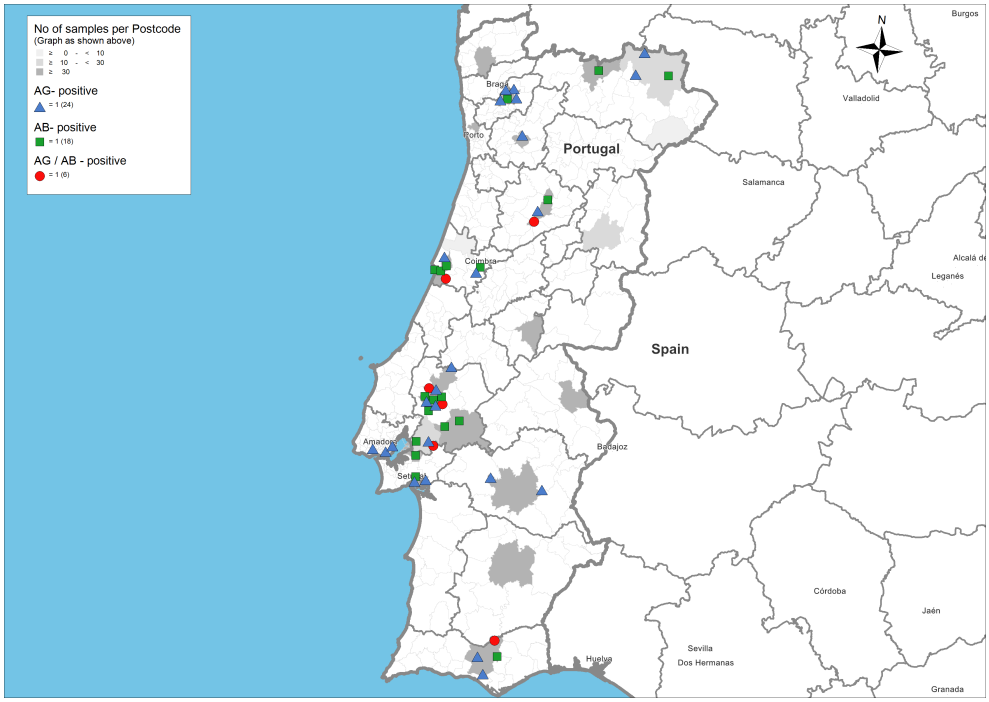

Table 1 - Serological results of 906 dog samples from Portugal tested for the presence of *Angiostrongylus vasorum* circulating antigens and for specific antibodies against *A. vasorum.*

	Positive samples (n)	%	95% confidence intervals
Antibody-positive	18	1.99	1.18-3.12
Antibody-positive only	12	1.32	0.68-2.30
Antigen-positive	24	2.65	1.70-3.92
Antigen-positive only	18	1.99	1.18-3.12
Antibody- and antigen-positive	6	0.66	0.24-1.43

4. Discussion

Positive dogs for both ELISAs were detected in the north, centre and southern areas of Portugal, indicating an active infection widely distributed throughout the sampled area. The endemic occurrence of *A. vasorum* in dogs from different geographical areas of Portugal is therefore confirmed. Interestingly, *A. vasorum* was nearly absent in the central-eastern part of the country that borders Spain. Howbeit, the lack of evidence of *A. vasorum* in certain regions of Portugal does not ensure its non-existence, and thus, geographical location should not be used as the unique criterion to suspect or rule out this diagnosis.

With 0.66% of the examined dogs being positive in both ELISAs, the prevalence in Portugal is apparently higher than that found for Germany (Schnyder et al., 2013a) or Poland (Schnyder et al., 2013b) and lower than in Hungary (Schnyder et al., 2015a), UK (Schnyder et al., 2013a) and Italy (Guardone et al., 2013), but not significantly. Nevertheless, it is important to highlight that this study surveyed shelter dogs, i.e., stray dogs usually not under any kind of prophylaxis and therefore frequently demonstrating higher parasitic prevalence (Alho et al., 2014).

Approximately 2% of the dogs were positive for specific antibodies against *A. vasorum*, indicating parasite exposure: these dogs may have been sampled between 3-6 weeks after an *A. vasorum* infection, when antigen detection is still negative (first positive results between 7-11 weeks after infection), or the dogs were parasite-free but still antibody-positive after anthelmintic treatment or natural clearance of the infection (Table 1) (Schnyder et al., 2015b).

The results herein presented confirm the endemic occurrence of *A. vasorum* in dogs from different geographical areas of Portugal and may support the hypothesis of a gradual progression of *A. vasorum* in Portugal, since the prevalence of this parasite in red fox necropsies have also increased, from 0.3% between 1970-1987 to 16.1% between 2000-2006 (Carvalho-Varela & Marcos 1993; Eira et al., 2006). Several recent studies from other European countries such as Germany, Hungary, Switzerland, Great Britain or Italy illustrate the highly successful establishment of *A. vasorum* in the last decades. Although the reasons for this impressive success are not fully understood, a similar success within Portugal cannot be excluded. The occurrence of *A. vasorum* is linked to the presence of final hosts, among which red foxes

represent powerful reservoirs and spreaders of this parasite, as indicated by higher prevalence in foxes compared to dogs (summarised in Koch & Willesen 2009). In fact, red foxes are the most widespread European wild canid (Otranto et al., 2015), greatly dispersed also all over the Iberian Peninsula (Macdonald & Reynolds 2008). This, in the absence of obvious geographic barriers, plays an important role in the expansion and establishment of *A. vasorum*, possibly explaining the high prevalence of this parasite detected in red foxes (*Vulpes vulpes)* in Portugal (Eira et al., 2006) and throughout Spain (Barbosa et al., 2005; Mañas et al., 2005; Gerrikagoitia et al., 2010). Also, the potential role of the wolf (*Canis lupus*) as a wild reservoir of *A. vasorum* can be mentioned based on a prevalence of 1.9-2.1% reported in wolves from Spain (Segovia et al., 2001; Segovia et al., 2007). Finally, the prevalence found in our study might be also explained by the results obtained in a pet owners' questionnaire performed in Portugal, where it was shown that although the majority of the owners give antiparasitic drugs to their dogs, this often occurs at irregular and consequently ineffective intervals, with only 11.8% of the dogs following the recommended endoparasitic treatment and only 28.4% uninterruptedly protected throughout the year from canine vector borne diseases (European Scientific Counsel Companion Animal Parasites (ESCCAP); Matos et al., 2015).

Interestingly, a simulation based on the observed distribution of *A. vasorum* in Europe and eco-climatic similarities predicted highly suitable areas in the north of Portugal and no suitability in the centre and southern part of the country (Morgan et al., 2009). Indeed, climate may play an important role in *A. vasorum* transmission, as the population dynamics and the activity of intermediate host species are highly dependent on temperature and moisture conditions. The north of Portugal is usually characterized by average low temperatures and high humidity in contrast to the south, where temperatures are frequently higher with lower humidity. Nevertheless, the slugs *Arion rufus* and *Deroceras laeve*, two gastropods known as intermediate hosts of *A. vasorum*, have already been described in distinct parts of the Portuguese territory (Grewal et al., 2003; Bank, 2011).

To conclude, positive cases detected in such distinct areas of Portugal suggest that this parasite is now widespread in endemic foci over nearly the whole country. Since there have been only few studies in Portugal, it is not clear if the epidemiological situation in the country is stable or if it is moving.

Despite the complexity and challenges involved in diagnosing *A. vasorum* infection, when early detection and prompt targeted therapy are undertaken, prognosis is good. Considering the impact of this disease on the health of affected dogs, it is thus important to increase knowledge concerning the epidemiological situation of this potentially fatal parasite. We believe that these results will be crucial to raise the awareness of veterinary practitioners, ensuring routine

screenings of lungworms in dogs, and a well-timed diagnosis and treatment. Furthermore, we hope this data will contribute to highlight the importance of owner's education in order to adopt behaviours that minimize the risk of infection.

Ethical standards

All institutional and national guidelines for the care and use of laboratory animals were followed.

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Chapter 2, Scientific publication 5

Seroprevalence of vector-borne pathogens and molecular detection of *Borrelia afzelii* in military dogs from Portugal

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Abstract

Background: Canine vector-borne diseases (CVBDs) are increasingly being reported worldwide and represent a serious threat to both animal and public health. Military dogs may constitute a risk group for the agents causing these diseases, as they frequently work outdoors in different areas and are thus exposed to vector arthropods. In order to assess the risk of exposure of this type of dogs, a serological and molecular survey was conducted in military working dogs in Portugal. One hundred apparently healthy dogs were surveyed. Serum samples were tested for antigens of *Angiostrongylus vasorum* and *Dirofilaria immitis*; and for antibodies to *A. vasorum*, *Anaplasma* spp., *Babesia* spp., *Ehrlichia canis*, *Leishmania infantum*, *Rickettsia* spp. and Toscana virus. Serum was tested by polymerase chain reaction for *Borrelia burgdorferi* sensu lato, with sequencing of the DNA products.

Results: Forty-nine per cent of the dogs were seropositive for antibodies against *Rickettsia* spp., 16% for *Anaplasma* spp., 13% for *L. infantum*, 7% for *E. canis*, 5% for *A. vasorum* (including 1% positive for both antibodies and circulating antigens), 3% for *Babesia* spp. and 1% positive for Toscana virus. *B. burgdorferi* s.l. was detected in 8 out of 94 dogs tested (8.5%) and in 3 cases (3.2%) nucleotide sequence analysis showed identity with the genospecies *Borrelia afzelii*. No positive cases were recorded for *D. immitis.* Overall, 66% of the dogs were positive for at least one out of the eight tested CVBD agents, six of which are zoonotic (i.e. *Anaplasma* spp., *Borrelia* spp., *E. canis*, *L. infantum*, *Rickettsia* spp. and Toscana virus). Serological specific antibody detection against more than one CVBD agent (including molecular detection of *Borrelia* spp.) was recorded in 25% of the dogs, comprising 19% with positive reaction to two agents, 5% to three agents and 1% to four agents.

Conclusions: These results reveal a high occurrence of CVBD agents in military working dogs in Portugal and highlight the need to maintain a comprehensive and regular prophylaxis to reduce the contact between working dogs and those pathogens. For the first time in Portugal, *B. afzelii* DNA was identified in dogs and a dog was found seropositive for antibodies against Toscana virus.

Keywords: Canine Vector-Borne Diseases, Dog, Epidemiology, Military, Seroprevalence, Portugal, *Borrelia afzelii*, *Borrelia burgdorferi* sensu lato, Toscana virus, Zoonosis.

1. Background

Canine vector-borne diseases (CVBDs) are an emerging problem worldwide due to their frequency, morbidity and zoonotic potential, representing a serious threat to animal and public health (Day, 2011). These diseases are caused by a wide range of pathogens, comprising viruses, bacteria, protozoa and helminths transmitted to dogs by different arthropods, namely ticks, fleas, mosquitoes and phlebotomine sand flies (Otranto, Dantas-Torres & Breitschwerdt, 2009). Several factors have been linked to the expansion of CVBDs, including an increased exposure to old and new infectious agents. Enhanced international commerce, faster and incremented global transport, deforestation and urbanization, abundance of wildlife hosts, demographic and political changes, climate alterations and drug resistance among vectors and pathogens, are making the spread of ectoparasites and their pathogens a no-boundary global event (Colwell, Dantas-Torres & Otranto, 2011).

Bacterial agents of CVBDs such as *Anaplasma platys* (the cause of infectious canine cyclic thrombocytopenia), *Anaplasma phagocytophilum* (granulocytic anaplasmosis), *Borrelia burgdorferi* sensu lato complex (Lyme disease), *Ehrlichia canis* (canine monocytic ehrlichiosis) and *Rickettsia conorii* (Mediterranean spotted fever) are tick-borne diseases of increasing concern (Otranto et al., 2014; Sainz et al., 2015; Solano-Gallego et al., 2015). Also important are some protozoal agents of CVBDs including *Babesia canis* and *Babesia vogeli* (canine babesiosis or piroplasmosis), also vectored by ticks (Solano-Gallego & Baneth, 2011), and *Leishmania infantum* (leishmaniosis), vectored by phlebotomine sand flies (Palatnik-de-Sousa & Day, 2011). Other relevant CVBD agents are the nematode *Dirofilaria immitis*, a mosquito-borne pathogen, which induces cardiopulmonary dirofilariosis or heartworm disease; and Toscana virus, which is an arbovirus (i.e. an arthropod-borne virus) vectored by phlebotomine sand flies (Charrel et al., 2005). Although not transmitted by arthropods but by slugs or snails, the nematode *Angiostrongylus vasorum*, also known as the "French heartworm" (causing canine angiostrongylosis), is an increasingly reported pathogen in Europe (Elsheikha et al., 2014). In general, canine infections with CVBD agents range from mild to severe and life-threatening forms. Clinical signs may include lethargy, weight loss, fever, lymphadenomegaly, poor appetite or anorexia, but are often variable and non-specific, thus requiring diagnosis to be complemented at the laboratory level. In addition, the vast majority of these miscellaneous pathogens (i.e. *Anaplasma* spp., *Borrelia* spp., *E. canis*, *L. infantum*, *R. conorii* and Toscana virus) have also zoonotic character, causing disease in humans, thus representing a great veterinary and public health threat.

Military working dogs (MWD), also known as police dogs, are specifically trained to assist security and law-enforcement personnel in their work. These animals make periodic fieldwork

in the most diverse climatic conditions of national and international territories, spending long periods outdoors, which increase the contact with wild animals and diverse types of vectors. The nature of their activities exposes them to risk factors distinct from those of common pet dogs, and may make them more susceptible to CVBDs (Davoust, Toga, Dunan & Quilici, 1994). Likewise, MWD have intense contact with people, as they are paired with a dog handler, i.e. someone who trains and is accompanied by the animal for long periods, a fact that increases the risk of transmission of zoonotic pathogens.

Little is known about the risk of MWD regarding CVBDs. Few studies have been conducted so far and no surveillance mechanisms are in place to assess prevalence or geographical range in Portugal and Europe (Vidal et al., 2014). Considering the emergence of CVBDs in Europe, as well as the lack of studies regarding CVBDs in MWD, a preliminary epidemiological study was conducted, involving serological and molecular testing of dogs, kept in military bases across continental and insular Portugal.

2. Methods

A survey was conducted with 100 MWD belonging to the Portuguese Air Force. Blood was collected in distinct air bases in mainland Portugal (districts of Aveiro, Beja, Leiria, Lisboa and Setúbal) and also on the Atlantic archipelagos of the Azores and Madeira (Fig. 1). Serum was obtained from each dog and stored at –20 ºC until use.

A complete record was kept for each sampled dog, including gender, age, breed and body condition. All dogs were apparently healthy with no clinical signs or historical abnormalities compatible with CVBDs. The dogs were housed outdoors. All animals received a combination tablet of praziquantel, pyrantel pamoate and febantel every 4 months (Drontal® Plus XL, Bayer Animal Health); a deltamethrin-impregnated collar every 4 months (Scalibor®, MSD Animal Health); an ivermectin tablet monthly (Heartgard®, Merial); and an imidacloprid and permethrin spot-on monthly (Advantix®, Bayer Animal Health).

Out of the 100 dogs tested, there were 92 males and 8 females. Age ranged from 7 to 132 months (median: 60 months) and average body condition was 4.9 (range: 1 to 9). Six breeds were represented: German Shepherd (n = 64), Labrador Retriever (n = 15), Belgian Shepherd (n = 16), Dutch Shepherd (n = 3), Rottweiler (n = 1) and Dobermann (n = 1).

To test for the presence of *D. immitis* circulating antigens, a rapid commercial qualitative antigen kit WITNESS® *Dirofilaria* (Synbiotics, Europe) was used. All procedures were performed as recommended by the manufacturer. Sera were tested by enzyme-linked immunosorbent assay (ELISA) for detection of specific antibodies to *L. infantum*, as described by Mettler, Grimm, Capelli, Camp & Deplazes (2005). Sandwich ELISAs were used for

detecting antibodies to *A. vasorum* (Schucan et al., 2012) and for the presence of *A. vasorum* circulating antigens (Schnyder, Tanner, Webster, Barutzki & Deplazes, 2011). Commercial immunofluorescent antibody tests (IFAT) were used to detect the IgG-antibodies to *Anaplasma* spp. (MegaScreen® FLUOANAPLASMA ph. kit), to *Babesia* spp. (MegaScreen® FLUOBABESIA canis kit), to *E. canis* (MegaScreen® FLUOEHRLICHIA c. kit) and to *Rickettsia* spp. (MegaScreen® FLUORICKETTSIA con. kit), according to the manufacturer's instructions (Megacor, Horbranz, Austria). Sera were tested for immunoglobulin G (IgG)-class antibodies against Toscana virus using an in-house IFAT; and samples with IgG titres of 32 were considered as positive.

For molecular detection of *B. burgdorferi* s.l., DNA extraction from serum samples was carried out using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) tissue protocol, with some modifications including a mechanical lysis and the addition of carrier DNA (Salmon Sperm DNA [Sigma, St. Louis, MO, USA] at a final concentration of 10 µg/µl). Sera were screened for the presence of *B. burgdorferi* s.l. DNA using a nested PCR targeting the 5S-23S (rrf-rrl) rRNA intergenic spacer region using the primers pairs (23SN1 and 23SC1 – outer primers; 23N2 and 5SCB – inner primers), as described by Rijpkema, Molkenboer, Schouls, Jongejan and Schellekens, 1995). The amplification reactions were performed in a C1000 thermocycler (Bio-Rad, Hercules, CA, USA) with an initial step at 94 ºC for 3 min, followed by 25 rounds of temperature cycling (94 ºC for 30 s, 52 ºC for 30 s, and 72 ºC for 1 min) for outer primers and 40 rounds of temperature cycling (annealing step of 54 ºC for 30 s) for inner primers and an ending step at 72 ºC for 7 min for both amplification reactions. A DNA solution extracted from *B. burgdorferi* s.l. culture and ultrapure water were used as positive and negative controls of amplification, respectively. The PCR amplified products were analysed by 1.5% agarose gel electrophoresis and DNA-positive samples were sequenced at StabVida (Caparica, Portugal) using internal PCR primers. Nucleotide sequence analysis and comparison with other relevant reference sequences were performed using the BLAST suite at GenBank®.

Whenever appropriate, the chi-square or Fisher's exact tests were used to compare proportions, and a probability (p) value < 0.05 was considered as statistically significant. Exact binomial 95% confidence intervals (CI) were established for proportions. Analyses were done using the StatLib and SPSS® 20 software for Windows.

3. Results

Forty-nine per cent of the dogs were seropositive for antibodies against *Rickettsia* spp., 16% for *Anaplasma* spp., 13% for *L. infantum,* 7% for *E. canis,* 5% for *A. vasorum* (including 1% positive for both antibodies and antigens), 3% for *Babesia* spp. and 1% positive for Toscana

virus. *B. burgdorferi* s.l. DNA was detected in 8 out of 94 dogs tested (8.5%) and in 3 cases (3.2%) sequence analysis showed identity with the genospecies *Borrelia afzelii*. No positive results were recorded for *D. immitis* antigen. Overall, 66% of the dogs were positive for at least one out of the eight tested agents of CVBD, six of which are of zoonotic concern (i.e. *Anaplasma* spp., *Borrelia* spp., *E. canis*, *L. infantum*, *Rickettsia* spp. and Toscana virus). In addition, single antibody positivity to *A. vasorum* was found in three dogs, accounting for an additional 3% prevalence. Serological specific antibody detection against single CVBD agents (including molecular detection of *Borrelia* spp.) was recorded in 41% of the dogs. Positive reactions to more than one CVBD agent (including *Borrelia* spp.) was recorded in 25% of the dogs, comprising 19% positive to two agents, 5% to three agents and 1% to four agents (Table 1).

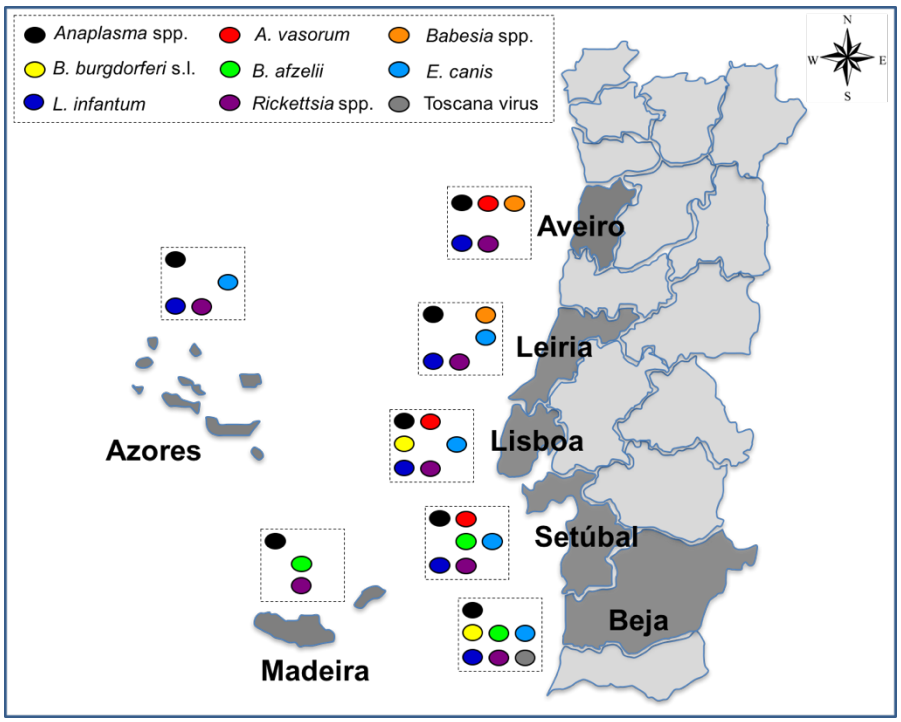
Table 1 - Serological specific antibody detection against vector-borne pathogens (VBP) in military working dogs from Portugal (including molecular detection of *Borrelia* spp.)

Agent(s)	Positive dogs		
	n	%	CI
Positive reaction against single VBP	41 [1]	41.0 [a,b,c]	31.3–51.3
Anaplasma spp.	5	5.0	1.6–11.3
Borrelia burgdorferi sensu lato	4 [2]	4.0	1.1–9.9
Ehrlichia canis	1	1.0	0.0–5.4
Leishmania infantum	3	3.0	0.6–8.5
Rickettsia spp.	28 [3,*]	28.0	19.5–37.9
Positive reaction against two VBP	19	19.0 [a,d,e]	11.8–28.1
Anaplasma spp. + *B. burgdorferi* s.l.	1 [2]	1.0	0.0–5.4
Anaplasma spp. + *E. canis*	2 [3]	2.0	0.2–7.0
Anaplasma spp. + *Rickettsia* spp.	5	5.0	1.6–11.3
Babesia spp. + *Rickettsia* spp.	1	1.0	0.0–5.4
B. burgdorferi s.l. + *Rickettsia* spp.	2 [2]	2.0	0.2–7.0
E. canis + *Rickettsia* spp.	2	2.0	0.2–7.0
L. infantum + *Rickettsia* spp.	6 **	6.0	2.2–12.6
Positive reaction against three VBP	5	5.0 [b,d]	1.6–11.3
Anaplasma spp. + *B. burgdorferi* s.l. + *E. canis*	1	1.0	0.0–5.4
Anaplasma spp. + *Rickettsia* spp. + *L. infantum*	1	1.0	0.0–5.4
Anaplasma spp. + *Rickettsia* spp. + Toscana virus	1	1.0	0.0–5.4
Babesia spp. + *L. infantum* + *Rickettsia* spp.	2 **	2.0	0.2–7.0
Positive reaction against four VBP	1	1.0 [c,e]	0.0–5.4
Anaplasma spp. + *E. canis* + *L. infantum* + *Rickettsia* spp.	1	1.0	0.0–5.4
Single + co-infections	66	66.0	55.8–75.2

[1] Including four dogs singly positive for *Angiostrongylus vasorum*; [2] including one DNA sequencing result of *Borrelia afzelii*; [3] including one dog positive for *A. vasorum*; *, ** three and one dogs not tested for *B. burgdorferi* s.l., respectively; [a,b,c] $p < 0.001$; [d,e] $p \leq 0.002$; CI: 95% confidence interval.

Positive animals were distributed over all sampled areas in the country and a southward trend of increased pathogen diversity was observed (Fig. 1). Sequences identical to *B. afzelii* were found in two dogs from Setúbal and Beja (99% identity GenBank® accession no. KU891495) and in a dog from Madeira (98% identity GenBank® accession no. KU891496). Sequencing results from the other samples were inconclusive due to low quality of the obtained sequences.

Figure 1 - Regional occurrence (presence or absence) of vector-borne pathogens and *A. vasorum* in military working dogs from the seven air bases in mainland Portugal (Aveiro, Beja, Leiria, Lisboa and Setúbal) and on the Atlantic archipelagos of Azores and Madeira.



No statistically significant associations were found for positivity to CVBD agents among the gender and age categories.

4. Discussion

Out of the tick-borne pathogens, *Rickettsia* spp. was the most prevalent, followed by *Anaplasma* spp., *E. canis*, *Babesia* spp. and *B. burgdorferi* s.l. In addition, *Rickettsia* spp. and *Anaplasma* spp. were detected in all the areas assessed, either in mainland or on insular regions. Besides, *L. infantum*-positive dogs were distributed throughout all the regions of mainland, and also on the Atlantic archipelago of Azores. For the first time, *B. afzelii* DNA was detected in dogs in Portugal, with this being a genospecies usually associated with small mammals and one of the causative agents of the most common tick-borne disease in Europe and North America. Additionally, one dog was found positive for antibodies to Toscana virus, indicating a previous exposure to this agent. Although to date there is no evidence that dogs can develop disease when infected with this virus, this cannot be excluded, as well as their potential as amplifying hosts in the Toscana virus cycle (Charrel et al., 2005). In addition, specific antibodies against *A. vasorum* were detected in 5% of the MWD, including one case simultaneously positive for *A. vasorum* antigen, which denotes an on-going infection. These data bring new information

concerning the *A. vasorum* presence and geographical distribution in Portugal, as only a few cases of infection are documented in dogs from Portugal (Alho et al., 2014; Alho et al., 2016). In the present study, a very high number of dogs were found to be positive for at least one pathogen, with two thirds of them being positive to at least one of the CVBD agents tested and/or *A. vasorum* (Table 1). In fact, co-infection is a frequent condition in dogs, since several arthropods are competent vectors of more than one pathogen and may share the same environment. This is the case of the brown dog tick, *Rhipicephalus sanguineus* sensu lato, known for its worldwide distribution, which serves as confirmed vector for *E. canis* and *R. conorii,* and as presumed vector for *A. platys* (Cardoso et al., 2015).

Co-infection is frequent all over Portugal, as previously evidenced by Cardoso, Mendão and Madeira de Carvalho (2012), where 14% of apparently healthy dogs and 46.3% of clinically suspect dogs were seropositive to at least one tested agent out of *Anaplasma* spp., *B. burgdorferi* s.l., *D. immitis*, *E. canis* and *L. infantum*. Similar findings were evidenced by Menn, Lorentz and Naucke (2010), in southern Portugal, where 87% of autochthonous shelter dogs were positive to at least one of the following: *A. phagocytophilum*, *B. canis*, *E. canis*, *H. canis*, *L. infantum*, *R. conorii* and microfilariae. In fact, Portugal is a country where several CVBDs are endemic, a situation which is partially explained by the mild Mediterranean climate that favours vector development and survival, which in turn contributes to justify the high prevalence levels detected in the present study. Additionally, many of these CVBD agents are of zoonotic concern with dogs serving as potential reservoirs or sentinels for wide variety of human infections. In fact, the close physical contact and daily interaction between military dogs and their handlers may increase the potential risk for the transmission of zoonotic pathogens. This is the case of *Anaplasma* spp., *B. burgdorferi* s.l., *E. canis*, *L. infantum*, *Rickettsia* spp. and Toscana virus, among others.

The prevalence detected in the present study may also represent the reality of MWD from military forces in other countries as they occasionally perform missions abroad. Previous studies conducted in MWD are few and punctual, and have shown a wide variation on the prevalence of canine vector-borne infections, mainly depending on the area under study, the diagnostic methods used and the ongoing prophylactic regimen (Vidal et al., 2014; Davoust, Roqueplo, Parzy, Watier-Grillot & Marié, 2013). In a serological study to assess the exposure of MWD to tick-borne pathogens in South Korea, seroprevalence for *Anaplasma* and *Ehrlichia* were 4.4% and 0.6% based on ELISA, and 24.7% and 22.5% based on IFAT, respectively, and 1.1% for *B. burgdorferi* s.l. based only on ELISA (Bell et al., 2012). In Spain (Madrid), out of 131 dogs from the National Police Department, 2.3% had antibodies to *E. canis* (Sainz, Tesouro, Rodriguez, Mayoral & Mazzucchelli, 1995). In Slovakia, out of the 710 police and

military dogs investigated for the presence of microfilariae in blood, 18% were diagnosed positive for *Dirofilaria* infection (Miterpáková et al., 2010); in New Caledonia, where canine dirofilariosis is endemic, a serological study revealed no positive results for *D. immitis* antigen in a population of MWD undergoing moxidectin prophylaxis (Watier-Grillot, Marié, Cabre & Davoust, 2011). Regarding *L. infantum*, a serological study performed in three MWD kennels in southeastern France showed a seroprevalence of 11.6% (Davoust et al., 1994).

It is important to keep in mind that the positive serological results presented is this study might be due to either an on-going infection or simply to a previous contact or exposure to the agent. For that reason, and whenever available and economically feasible, serological screenings should be complemented with molecular-based detection methods to ascertain on whether infections are active or not (Cardoso et al., 2015). Likewise, serological cross-reactivity could occur between pathogens and thus PCR would be an advantage to achieve an accurate etiological diagnosis and to establish which species are implicated and circulating in the population. Yet, it must be emphasized that despite the tight prophylactic regimen implemented in this MWD, exposure to multiple CVBD agents was observed among this canine population, suggesting they should be regarded as a risk group. In spite of that, no dog showed any clinical signs. This is quite relevant as they can act as "silent" reservoirs and sentinels, fostering the perpetuation and transmission of endemic or exotic pathogens among other animals. Furthermore, subclinically infected dogs can transport arthropods harbouring pathogens into close proximity to people or even serve as a "direct" reservoir for human vector-borne infections, as several of these CVBDs have a zoonotic impact (Shaw, Day, Birtles & Breitschwerdt, 2001). Also considering the impact of these diseases on the health of dogs, it is thus crucial to increase knowledge concerning their epidemiological situation and ensure routine screening. The results herein presented are essential to a better understanding of the potential CVBDs in this peculiar population. These new data will be useful for both medical and veterinary services engaged in the control of vector-borne diseases under the scope of One Health, and will serve as a reference for future research, prevention and control actions.

5. Conclusions

In terms of the tested pathogens, this is the most comprehensive study carried out to assess the exposure of MWD to agents of CVBDs worldwide, and presents the first report of a seropositive dog for Toscana virus in Portugal, as well as the first time *B. afzelii* DNA has been identified in dogs in the country. Although these animals have daily monitoring, balanced nutritional support, regular medical care, tight prophylaxis and anti-parasitic control, their activities seem to steadily increase their contact with CVBD agents. Taking into account their long periods of

work outdoors (both day and night) and their high mobility, these dogs are at a high risk of exposure to vectors and of contact with other domestic and wild animals, thus acting as a sentinel population. Considering that many of these CVBD agents are of significant zoonotic concern, an integrated approach under the scope of "One World, One Health" should be put in practice to control pathogens and promote higher animal and public health standards. Further epidemiological studies are needed to improve scientific knowledge and risk assessment concerning MWD and CVBDs.

Ethics approval

All the clinical procedures in this study were in accordance with Portuguese (Decree-Laws no. 314/2003 and no. 113/2013) and European legislation for the protection of animals.

Authors' contributions

AMA conceived the study, performed serological analysis and drafted the manuscript; JP conducted clinical examination and sample collection; AA performed molecular analysis for *Borrelia* spp.; FA performed serological analysis for Toscana virus; MS helped with the serological analysis for *A. vasorum* and contributed with data analysis; FG helped with the serological analysis for *L. infantum* and contributed with data analysis; ACC organized logistics and conducted sample collection; LC analysed data and revised the manuscript; PD reviewed the manuscript; LMC conceived the study and reviewed the manuscript. All authors read and approved the final manuscript.

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CHAPTER 3

Epidemiological survey of *Dirofilaria immitis* and *Angiostrongylus vasorum* in wild carnivores in Portugal

Chapter 3, Scientific publication 1

Serological survey of the heartworms *Dirofilaria immitis* and *Angiostrongylus vasorum* in red foxes (*Vulpes vulpes*) from Portugal

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Abstract

Background: *Dirofilaria immitis* and *Angiostrongylus vasorum* are two major heartworms of canids, whose adult stages reside in the pulmonary arteries and right chambers of the heart, causing a potentially fatal illness in dogs. Wild canids may constitute a potential reservoir for both parasites. In Portugal, despite the fact that *D. immitis* and *A. vasorum* have been reported in dogs, little is known about their occurrence in red foxes (*Vulpes vulpes*), a known host for heartworms. The present survey aimed at investigating the prevalence and distribution of *D. immitis* and *A. vasorum* in vulpine populations from Portugal by serological analysis, to assess the potential epidemiological role of these canids as reservoirs of both infections in the country.
Methods: Sera or muscle juice were obtained from 118 wild red foxes shot during the official hunting season or killed by traffic accidents, between 2008 and 2010. These animals came from eight districts of Portugal, belonging to two main geographical areas: North (Aveiro, Braga, Bragança, Porto, Viana do Castelo and Vila Real) and South (Évora and Setúbal). A rapid commercial qualitative antigen kit (WITNESS® *Dirofilaria*) was used to test for *D. immitis* circulating antigens. Two sandwich enzyme-linked immunosorbent assays (ELISA) were used for detecting circulating antigens of and antibodies against *A. vasorum*.
Results: Overall, 8.5% (10/118) were positive to *D. immitis* antigen test, with positive animals found in the northern (Braga, Bragança, Viana do Castelo and Vila Real) and southern (Évora) areas. In addition, 12.7% (15/118) foxes were positive for *A. vasorum*, including 5.9% (7/118) positives for both *A. vasorum* antigen and antibody detection, 5.1% *A. vasorum* antigen-positive only and 1.7% *A. vasorum* antibody-positive only. Positives animals were detected in the North (Bragança, Viana do Castelo and Vila Real) and South (Évora). No statistically significant differences were found either for *D. immitis* or *A. vasorum* (double positive results) regarding geographical area, gender or age.
Conclusions: This is the first serological study performed in Portugal in wild carnivores to assess the prevalence of canine heartworms. Infections with *D. immitis* and *A. vasorum* have been demonstrated as prevalent and widely distributed in populations of red foxes in Portugal.

Keywords: Epidemiology, ELISA, *Dirofilaria* antigen test, Heartworms, Red fox, *Vulpes vulpes*, *Dirofilaria immitis*, *Angiostrongylus vasorum*, Portugal.

Chapter 3, Scientific publication 2

Dirofilaria immitis in pinnipeds and new host record

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Abstract

Background: *Dirofilaria immitis* is a mosquito-borne pathogen that is spreading worldwide, and the associated infection (i.e. dirofilariosis) is becoming a threat to animals and humans living in endemic areas. Little is known about the occurrence and risk of infection of *D. immitis* in pinnipeds. Here we report dirofilariosis by *D. immitis* in several pinniped species kept in captivity in Portugal.

Methods: Animals were housed in an oceanographic park located in Algarve, southern Portugal, a geographical area endemic for canine dirofilariosis. To assess the occurrence of *D. immitis*, blood was collected from the park's resident pinniped population, which consisted of 16 animals (5 common seals *Phoca vitulina*, 2 grey seals *Halichoerus grypus*, 3 California sea lions *Zalophus californianus* and 6 South African fur seals *Arctocephalus pusillus pusillus*). *Dirofilaria immitis* nematodes were detected by real-time PCR and by the presence of circulating antigens. In addition, modified Knott's technique was performed to detect circulating microfilariae. Necropsies and histopathological examination of two animals which died during the study were also conducted.

Results: Out of the 16 pinnipeds housed at the park, seven (43.8%) were positive for *D. immitis* by real-time PCR (3 *P. vitulina*, 2 *Z. californianus* and 2 *A. p. pusillus*), two of which (*P. vitulina*) were also positive for the nematode's antigen. Additionally, *D. immitis* microfilariae were detected in one *A. p. pusillus*. Furthermore, several *D. immitis* specimens were retrieved from the right ventricle and pulmonary arteries at the necropsy of one *P. vitulina* and one *A. p. pusillus*.

Conclusions: This study provides new epidemiological data on *D. immitis* infection in pinnipeds diagnosed through clinical, molecular and pathological findings. Additionally, the South African fur seal is herein reported as a new host for this zoonotic filarioid. The situation herein described could also occur in other parks located in areas where canine dirofilariosis is endemic. Active surveillance and preventive measures of dirofilariosis in pinnipeds on a local and global scale are therefore vital to improve the early diagnosis and control of dirofilariosis.

Keywords: *Dirofilaria immitis*, Pinnipeds, South African fur seal, Dirofilariosis, Vector-borne disease, Wildlife, Zoonosis.

1. Background

Dirofilaria immitis (Spirurida: Onchocercidae) is a mosquito-borne pathogen spreading worldwide, and the associated infection (i.e. dirofilariosis) is becoming a threat to animals and humans living in endemic areas (Simón et al., 2012). Although definitive hosts are primarily domestic and wild canids, *Dirofilaria immitis* shows low vertebrate host specificity, infecting several mammalian species (e.g. black bears, cats, ferrets, lions, otters, ocelots). In humans, this parasite may cause a severe clinical condition of increasing concern, with adult stages located mostly in the patient's lungs, eyes or other anatomical districts (Simón et al., 2012). However, little is known about the occurrence and risk of infection of *D. immitis* in pinnipeds. Only a few cases of infection in captive pinnipeds have been described so far (King, 1964; Forrester, Jackson, Miller & Townsend, 1973; Medway & Wieland, 1975; White, 1975; Sato et al., 2002; Van Bonn, 2015). Accordingly, no epidemiological studies on pinniped populations are available (Krucik, Van Bonn & Johnson, 2016) and adult *D. immitis* have only been found in one hooded seal (*Cystophora cristata*) (King, 1964), one common seal (*Phoca vitulina*) (Medway & Wieland, 1975), and in California sea lions (*Zalophus californianus*) kept in zoological parks in Florida (Forrester et al., 1973), Louisiana (White, 1975) and Japan (Sato et al., 2002). In the above-mentioned reports, nematodes were found upon necropsy in the right ventricle of the heart, pulmonary arteries, vena cavae, portal vein and pericardial sac (Forrester et al., 1973; Medway & Wieland, 1975; White, 1975; Sato et al., 2002). Clinical signs, only documented in California sea lions, included cardiopulmonary impairment, coughing and laboured breathing (Forrester et al., 1973; White, 1975). Indeed, pinnipeds might remain asymptomatic, even when large numbers of parasites inhabit their heart and associated vessels (Forrester et al., 1973; Medway & Wieland, 1975; White, 1975; Sato et al., 2002). Here we report dirofilariosis in a population of pinnipeds housed at an oceanographic park in Portugal and the South African fur seal, *Arctocephalus pusillus pusillus*, as a new host for *D. immitis*.

2. Methods

In 2013 and 2014, during the necropsy examinations of two adult South African fur seals (*A. p. pusillus*) housed at the Zoomarine park in Albufeira (Algarve, southern Portugal), *D. immitis* nematodes were accidentally found in the pulmonary arteries and right ventricle of two animals (animals A and B). These cases prompted an epidemiological survey to assess the occurrence of *D. immitis* in the park's resident pinniped population (n = 16). In 2015, 5 common seals (*Phoca vitulina*), 2 grey seals (*Halichoerus grypus*), 3 California sea lions (*Zalophus californianus*) and 6 South African fur seals (*A. p. pusillus*) were surveyed for *D. immitis* infection. All animals were housed in facilities with pools and dry areas and no ecto- or

endoparasitic treatments were administered. The animals originated from either Europe or Canada, and remained in Portugal for at least 10 years.

Physical examination was performed to check for the presence of abnormal clinical signs in individual animals. Blood was collected from the epidural intervertebral vein in the phocids (*P. vitulina* and *H. grypus*) and from the interdigital vein of the hind flippers or caudal gluteal vein in the otariids (*Z. californianus* and *A. p. pusillus*), as previously described (Gulland, Haulena & Dierauf, 2001). A rapid commercial qualitative antigen WITNESS® HW Heartworm Antigen Test Kit (Zoetis, Europe) was used to assess the presence of *D. immitis* circulating antigens and a modified Knott's technique was performed to detect circulating microfilariae in the pinnipeds' blood. In one of the animals, it was possible to perform an ultrasound to assess the presence of heartworm infection and evaluate cardiac function.

During this epidemiological survey two animals died and necropsies were conducted as described in (Geraci & Lounsbury, 2005; Pugliares et al., 2007). Lung and liver samples were collected for histopathological examination, fixed in 10% buffered formalin and embedded in paraffin; sections (3 μm thick) were stained with haematoxylin and eosin for routine microscopic examination.

Genomic DNA was extracted from segments (about 10 mm) of the adult worms collected from the necropsies and from the 16 blood samples, using a commercial kit (DNeasy Blood & Tissue Kit, Qiagen, GmbH, Hilden, Germany) and tested by real-time PCR (qPCR), based on SsoFast™ EvaGreen®, targeting partial cytochrome c oxidase subunit 1 (cox1), coupled with melting-curve analysis for the detection and discrimination of *Dirofilaria* spp. (Latrofa et al., 2012). The real-time PCR products were purified using Ultrafree-DA columns (Millipore, Bedford, USA), sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc.) in an automated sequencer (ABI-PRISM 377; Applied Biosystems Inc.). All sequences generated were compared to sequences available in GenBank using Basic Local Alignment Search Tool (BLASTn) (Altschul et al., 1997).

3. Results

Two *P. vitulina* were antigen positive (12.5%) and one *A. p. pusillus* scored positive for *D. immitis* microfilariae (6.3%) (Table 1). Circulating microfilariae were 300–305 μm long bearing a conical anterior edge and straight rear end (Fig. 1).

Table 1 - Test results of the 16 pinnipeds surveyed to *Dirofilaria immitis*.

Animal	Species	Gender	Country of origin	Birth year	Antigen test	Knott test	qPCR	Signs
1	*P. vitulina*	M	Portugal	2012	-	-	-	-
2	*P. vitulina*	F	Canada	1989	Positive	-	Positive[a]	-
3	*P. vitulina*	M	Canada	1996	-	-	-	-
4	*P. vitulina*	F	Portugal	2007	-	-	Positive	-
5	*P. vitulina*	F	Canada	1989	Positive	-	Positive	-
6	*H. grypus*	M	Portugal	2002	-	-	-	-
7	*H. grypus*	F	Canada	1990	-	-	-	-
8	*Z. californianus*	M	Portugal	1996	-	-	Positive	-
9	*Z. californianus*	M	Spain	2003	-	-	-	-
10	*Z. californianus*	M	Belgium	1996	-	-	Positive	-
11	*A. p. pusillus*	M	Portugal	1998	-	-	Positive	-
12	*A. p. pusillus*	M	United Kingdom	1992	-	Positive	Positive	Cough, lethargy and exercise intolerance
13	*A. p. pusillus*	M	Sweden	2002	-	-	-	-
14	*A. p. pusillus*	M	Sweden	2002	-	-	-	-
15	*A. p. pusillus*	M	Portugal	1996	-	-	-	-
16	*A. p. pusillus*	M	Spain	1996	-	-	-	-
Total		12M/4F			2	1	7	-

Abbreviations: F female, M male.
[a]Animal in which transthoracic echocardiography was performed revealing the presence of linear mobile hyperechoic structures within the right ventricle and main pulmonary artery, consistent with heartworms.

Figure 1 - Microfilaria of *Dirofilaria immitis* detected using the modified Knott's technique (scale-bar: 50 µm).

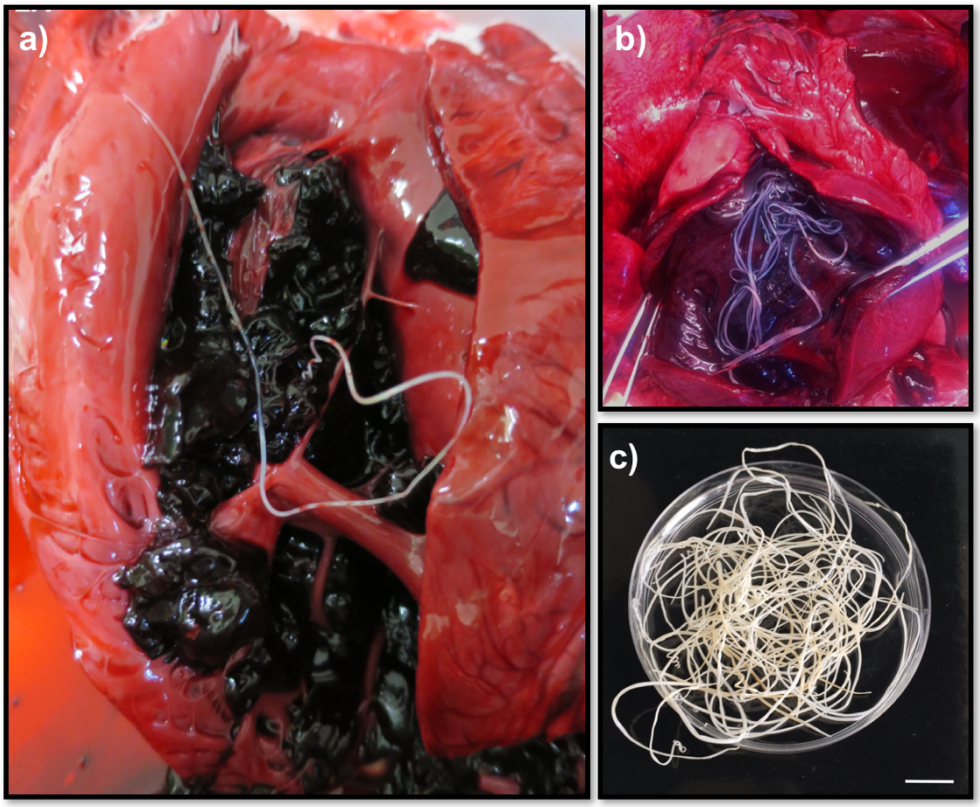
Seven (43.8%) out of the 16 animals were positive for *D. immitis* at qPCR (3 *P. vitulina*, 2 *Z. californianus* and 2 *A. p. pusillus*), with melting peaks (mean Tm = 75 °C) corresponding to the species-specific range of *D. immitis* positive control (mean ± SD: 75.7 ± 0.3 °C) (Table 1). One *P. vitulina* (Table 1, animal no. 5) and one *A. p. pusillus* (Table 1, animal no. 12) died during the study (Table 2). Data from these two animals were gathered with those obtained from animals' A and B dead prior to the epidemiological survey.

Table 2 - Data of the four necropsies of seals in which *Dirofilaria immitis* nematodes were detected.

Animal	Species	Gender	Birth year	Death year	Number of *D. immitis* adult nematodes	Location of adult nematodes	Pathological observations and histologic abnormalities
A	*A. p. pusillus*	M	1988	2013	15	Right ventricle and pulmonary artery	Severe pulmonary haemorrhages; extensive pulmonary congestion and moderate pulmonary emphysema
B	*A. p. pusillus*	M	1988	2014	10	Pulmonary artery	Moderate interstitial and exudative pneumonia; catarrhal bronchitis; moderate pulmonary congestion; central lobular hepatic congestion and dilation of the sinusoids
5	*P. vitulina*	F	1989	2016	32	Right ventricle and pulmonary artery	Extensive pulmonary congestion
12	*A. p. pusillus*	M	1992	2016	26	Right ventricle and pulmonary artery	Extensive pulmonary congestion

Overall, during the necropsies of these four animals (A, B, no. 5 and no. 12), adult male and gravid female nematodes were retrieved from the right ventricle and pulmonary arteries (Fig. 2).

Figure 2 - Adult nematodes of *Dirofilaria immitis* collected at the necropsies of South African fur seals. a) Adult nematode in the right ventricle. b) Adult nematodes (arrow) in the pulmonary artery, showing extensive pulmonary congestion. c) Male and female adult nematodes recovered from the blood clot (scale-bar: 2 cm).



Macroscopically, lungs were congested and haemorrhagic, and mild right ventricular hypertrophy was noticed. In these four cases, gross and histopathological abnormalities associated with *D. immitis* infection were present, including pulmonary congestion and haemorrhages (Fig. 3), pulmonary emphysema, interstitial and exudative pneumonia, catarrhal bronchitis and hepatic congestion (Table 2, Fig. 4). In the four cases, cardiopulmonary impairment was noticed. The adult nematodes collected from the four individuals were morphologically and molecularly identified as *D. immitis*. All cox1 gene sequences obtained from the adult nematodes and from the pinniped's blood were identical (GenBank accession number KX372755), showing 100% nucleotide identity to a *D. immitis* sequence in GenBank (KF553638).

Figure 3 - Macroscopic appearance of the lungs in the necropsy of a South African fur seal, highlighting an extensive pulmonary congestion and pulmonary haemorrhages.

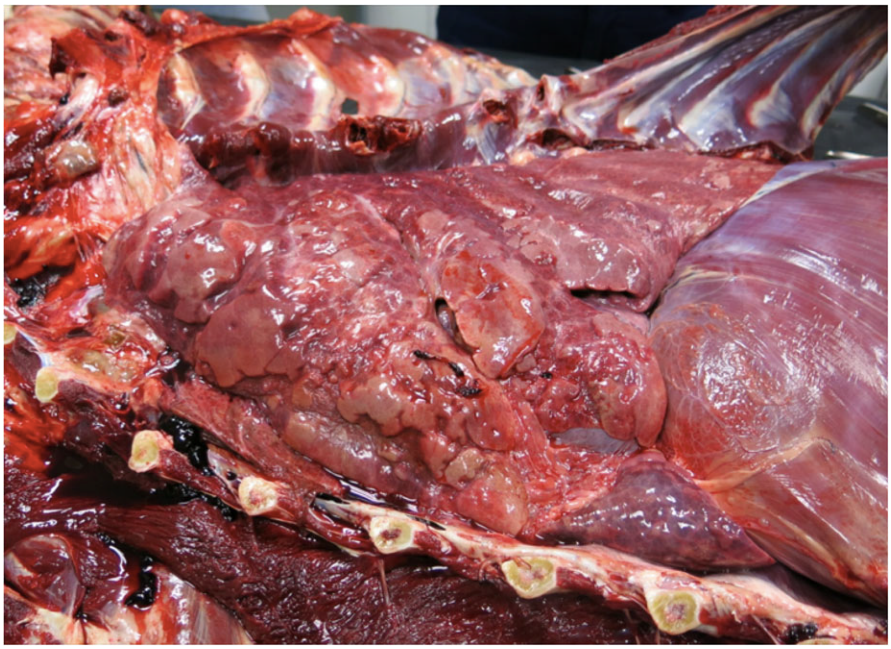
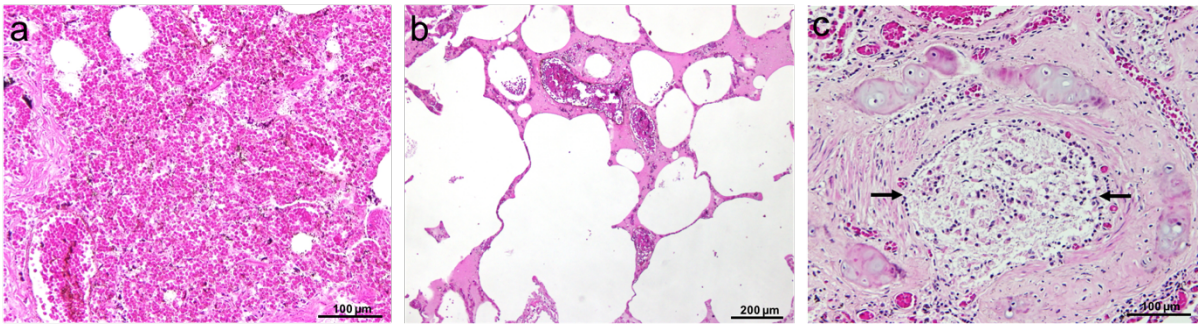


Figure 4 - Histopathology of the lungs of a South African fur seal, stained with haematoxylin and eosin: a) Severe pulmonary haemorrhages, note the presence of blood in the lumen of the alveoli. b) Extensive pulmonary emphysema. c) Catarrhal bronchitis, note the lumen of a bronchus obstructed with mucus (arrows).



4. Discussion

This study provides new epidemiological data of dirofilariosis by *D. immitis* in pinnipeds, diagnosed through clinical, molecular and pathological findings. The South African fur seal is herein described as a new host of *D. immitis*. All animals were housed in a tourist attraction (i.e. an oceanographic park) in the Algarve region, southern Portugal, an area that records the highest number of days/year with suitable conditions for *Dirofilaria* transmission (Alho et al., 2014), and the highest prevalence of canine dirofilariosis in mainland Portugal (Cardoso, Mendão & Madeira de Carvalho, 2012).

For the first time, qPCR was successfully used to diagnose *D. immitis* infection in pinnipeds. Indeed, qPCR detected seven infected animals, of which two *P. vitulina* and one *A. p. pusillus*

which were also positive for circulating antigen and microfilariae, respectively. qPCR was highly sensitive in diagnosing *D. immitis* in pinnipeds, since it was able to detect one animal that was only antigen-positive (no. 5) and another that was only microfilaremic (no. 12), although both presented several nematodes (including gravid females) during the necropsy.

In addition, it detected also another case (animal no. 2) only positive for the antigen, but in which transthoracic echocardiography revealed linear hyperechoic structures consistent with heartworms within the right ventricle and main pulmonary arteries. Furthermore, qPCR was also able to detect four other animals that were negative in all other diagnostic tests used (microfilariae and/or antigen; Table 1). All pinnipeds were retested by modified Knott's technique and antigen test to rule out potential false negatives. These further analyses displayed identical results. Positivity to qPCR suggests that alive parasites have been in contact with pinnipeds with no information on the current infection status. Indeed, this molecular assay may detect *D. immitis* DNA from a current infection or, theoretically, from a recently cleared infection. In addition, dirofilariosis in pinnipeds may be featured by transient and low intensity microfilaremia, as in the case of infection in cats. This might be the reason for the detection of microfilaremia in only one of the two pinnipeds who had male and female nematodes at necropsies. Additionally, rapid commercial *D. immitis* antigen tests were specifically developed for canine and feline blood samples, thus, their sensitivity and performance might be poor when used in pinnipeds, underestimating the true prevalence.

Although pinnipeds are aquatic mammals, they spend large periods of their life in terrestrial environments, and are therefore exposed to mosquito bites. As in the present survey, *D. immitis* could also occur in other parks in countries with endemic areas for canine dirofilariosis. The occurrence of this situation in the Algarve region, a popular summer destination, should be carefully considered due to the zoonotic potential of this parasite. Indeed, although human dirofilariasis has been often underdiagnosed (probably due to the lack of awareness amongst health professionals and to the difficulties in parasite identification), two cases of pulmonary nodules by *D. immitis* (Araújo, 1996) and two cases of subcutaneous dirofilariasis by *Dirofilaria repens* (Rombert, Nunes, Azevedo & Sinari, 1992; Baptista-Fernandes et al., 2015) have already been reported in Portugal.

5. Conclusions

This study emphasizes the need for active surveillance of dirofilariosis in facilities where animals and humans are in close contact, and strengthens the need for routine heartworm preventive measures (Otranto et al., 2013) and vector control strategies. The high prevalence of *D. immitis* herein reported in a confined area where pinnipeds are kept, may represent a risk

interface for zoonotic pathogen transmission. Therefore, a One Health approach applied on a local and global scale (Schwind et al., 2014) is vital to improve early diagnosis and control of zoonotic pathogens in humans and wildlife.

Abbreviation

qPCR: real-time PCR

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Availability of data and materials

All data generated or analysed during the study are included in the article.

Authors' contributions

AMA and IM conceived the study, performed morphological and serological analysis and drafted the manuscript; VC performed morphological and molecular analysis and drafted the manuscript; CF and NS conducted clinical examination, sample collection and necropsies; JJC performed histopathological analysis; MSL performed molecular and data analysis; DO analysed data and revised the manuscript; LMC conceived the study and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval

All technical procedures were reviewed and approved by Zoomarine's Ethical and Animal Welfare Committee. The protocol was performed in accordance to the standards and guidelines of the Alliance of Marine Mammal Parks and Aquariums (AMMPA) and European Association of Aquatic Mammals (EAAM), and to the National legislation regarding animal welfare (DL 276/2001 and DL 314/2003).

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CHAPTER 4

DNA detection of the endosymbiont *Wolbachia pipientis* in *Dirofilaria* infected dogs in Portugal

Chapter 4, Scientific publication 1

Detection of *Wolbachia* in *Dirofilaria* infected dogs in Portugal

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* The author participated in the field work, conducted clinical examination, sample collection, direct and serological analysis and co-produced the manuscript.

Abstract

Wolbachia pipientis, an intracellular endosymbiont bacteria of filarial nematodes, has been implicated in the pathogenesis of filarial diseases, in particular in heavy *Dirofilaria* spp. infections. Antibiotic therapy (doxycycline) against *Wolbachia* has been proven to be suitable adjunct therapy, prior to adulticide treatment of canine dirofilariosis. Despite its importance, investigation on the *Wolbachia/Dirofilaria* complex in Portugal had not been undertaken so far. This study reports the first detection of *Wolbachia* in *Dirofilaria* spp. infected dogs in the context of an ongoing epidemiological survey in central-south regions in the country. *Wolbachia* DNA was detected by PCR in 52.6% (20/38) of canine blood samples positive for *Dirofilaria immitis* based on parasitological (Knott's and Acid Phosphatase) and serological (Witness®*Dirofilaria*) methods. No *Wolbachia* DNA could be detected in samples from dogs with occult infections (parasite negative but antigen positive). The lack of *Wolbachia* detection in some microfilaremic dogs was somewhat unexpected and needs to be elucidated in further studies, as the presence or absence of these bacteria in association with microfilaria is of importance for veterinarians in the management and control of canine dirofilariosis.

Keywords: *Dirofilaria*, *Wolbachia*, Dogs, PCR, Portugal.

1. Introduction

Many studies have recently been conducted on *Wolbachia pipientis*, an intracellular bacterium of the Anaplasmataceae family and, so far, the only species of the genus (Merçot & Poinsot, 2009). First discovered in arthropods, where they are widespread, it can also be found in some nematodes, in particular in agents of human and animal Onchocercidae, such as *Onchocerca volvulus*, *Wuchereria bancrofti* and *Dirofilaria immitis* (Martin & Gavotte, 2010; Slatko, Taylor & Foster, 2010; Ferri et al., 2011).

Although little is known about the interaction between *W. pipientis* and their nematode hosts, available evidence suggests that it plays an essential role in the biology of *Dirofilaria* (Genchi, Kramer, Sassera & Brandi, 2011). In particular, filarial worms seem to need the bacteria to complete their life cycle or for embryogenesis. Therefore, the use of antibiotics (doxycycline) as an adjunct therapy targeting *W. pipientis* prior the administration of antiparasitic treatment, has been proven to contribute to the significant reduction in pathological side effects often induced by filaricidal drugs, particularly in heavy canine *Dirofilaria* infections (Grandi et al., 2010).

As a result, antibiotic therapy became a useful approach for treatment and control of filariosis (Genchi et al., 2011; McHaffie, 2012).

Whilst *W. pipientis* has been found in all studied adult *Dirofilaria* spp. worms, two recent studies on canine dirofilariosis produced contradictory results. Rossi et al. (2010) detected *W. pipientis* DNA in all microfilaremic dogs infected with *D. immitis* in Brazil, but Tabar, Altet, Martínez and Roura (2013) found *W. pipientis* DNA in only 30.6% of microfilaremic dogs in the Mediterranean.

The present study aims to elucidate the relationship between *D. immitis* and *W. pipientis* in canine populations in Portugal in order to improve approaches for the control of animal dirofilariosis.

2. Materials and methods

Blood samples were collected from 308 dogs from kennels in three districts of central Portugal (Setúbal, Santarém and Coimbra), from October to November 2011. The sample included 183 females and 125 males, ranging from 6 months to 16 years (median 4.9 years ± 3.3 SD) most of them mixed breed.

The presence of *D. immitis* circulating adult female antigen was tested using a commercial kit (Witness® *Dirofilaria*, Synbiotics). Blood microfilariae were detected and identified through Knott's modified and Acid Phosphatase techniques (Genchi, Venco & Genchi, 2007).

DNA was extracted from blood pellets (from Knott's technique) using the CTAB (Cetyltrimethylammonium bromide) method, adapted from (Stothard, Hughes & Rollinson, 1996). Briefly, 50 µl whole blood was incubated with CTAB buffer and 0.2 mg Proteinase K (Bioline) at 56ºC for 2 h, with agitation. DNA precipitation was done with 0.6 ml absolute ethanol and the pellet hydrated in 50 µl TE buffer (pH 7.0).

Wolbachia pipientis DNA was detected by PCR primers wolbF and wolbR, which amplify a fragment of 1018 bp of the 16S rRNA gene (Foster et al., 2008). PCR reactions were carried out with Illustra^{TM} puReTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK), using a final volume of 25 µl and a primer concentration of 1 µM and 2 µl DNA per reaction. PCR conditions were as in Foster et al. (2008) but with an annealing temperature of 56ºC. PCR sensitivity was tested with serial dilutions (by a factor of 10) of DNA from a female adult worm of *D. immitis* and from canine blood samples with known concentrations of <5 (n = 2) or >20 (n = 2) microfilariae per 20 µl of blood. PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and visualized under UV light. PCR products were purified with QIAquick Gel Extraction Kit (Qiagen) and sequenced on both strands by Stab Vida (Portugal). Sequences were edited and aligned using BioEdit (Hall, 1999) and compared to other similar sequences available in Genbank, as identified through BLAST.

Positive controls included DNA from a female *D. immitis* adult worm and DNA from *Drosophila melanogaster* known to harbour *W. pipientis* (by PCR).

Ethical considerations

The study was approved by the Commission on Ethic and Animal Welfare of the Faculty of Veterinary Medicine, Universidade Técnica de Lisboa, and all procedures were performed according to national and European legislations.

3. Results

Out of the 308 canine blood samples analysed, 47 samples (15.3%) were positive for *D. immitis* by at least one of the three diagnostic tests used (Knott's, Acid Phosphatase and Witness® *Dirofilaria*). *Dirofilaria immitis* antigen was detected in 33 samples (10.7%), while microfilariae were detected in 38 (12.3%) samples. Twenty-four samples (7.8%) where positive for both parasitological and serological tests whereas 14 (5.1%) cases were only positive for blood microfilariae and *D. immitis* antigen was exclusively detected in nine (3.3%) samples (Table 1).

Table 1 - Correlation of *D. immitis* detection by Knott's and Witness® *Dirofilaria* tests.

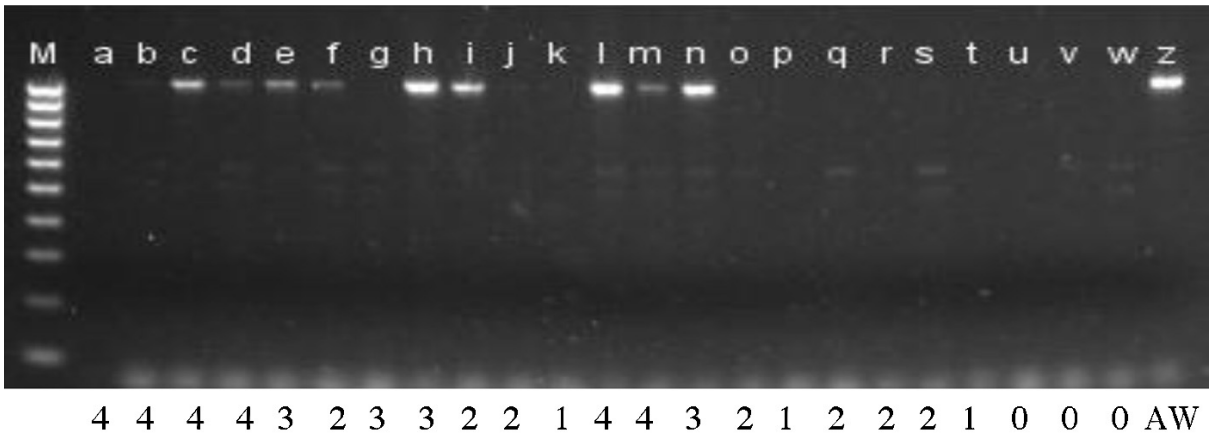
Techniques	**Witness® *Dirofilaria***		
	Ag+	**Ag−**	**Total**
Knott			
Mf+	24	14	38
Mf−	9	261	270
Total	33	275	308

Note: Mf, blood microfilariae; Ag, *D. immitis* antigen.

Microfilariae were identified as *D. immitis* in 37 of the 38 microfilaremic dogs by Acid Phosphatase (AP) technique; one positive blood sample (Knott's) failed to stain hampering species identification.

For *W. pipientis* DNA detection, a PCR with a sensitivity to detected down to 14 pg of DNA from adult female *D. immitis*, and 0.16 ng or 3.7 ng of DNA from canine blood with >20 and <5 microfilariae per 20 μl of blood, respectively, was used. The estimated detection limit was approximately 0.01–0.05 microfilariae per reaction mixture (Fig. 1).

Figure 1 - Examples of PCR detection of *Wolbachia* DNA in blood samples of dogs from Portugal.



Note: Lanes a–w, analyzed blood samples. Lane z, *D. immitis* adult worm DNA (positive control). Lane M, 100 bp DNA marker. Numbers at the bottom of the gel image correspond to the number of microfilariae (Mf) detected per 20 μl of blood: 4 = >20 Mf; 3 = 5–20 Mf; 2 = <5 Mf; 1 = Mf-negative/Ag-positive; 0 = negative sample.

In the 47 *Dirofilaria* positive samples, *Wolbachia* DNA was detected by PCR in 20 samples (42.6%), all of which from microfilaremic dogs (Fig. 1). Negative PCR reactions were confirmed by using higher and lower DNA dilutions than in the initial reaction.

The 16S PCR product obtained from two blood samples was sequenced (accession numbers HG328332 and HG328333) and found to have 100% identity (99% query coverage) to sequences attributed to *Wolbachia* sp. symbionts of nematodes (GenBank AF304445.1, AY652762.1 and Z49261.1).

4. Discussion

This survey of dogs in kennels in central Portugal found a global prevalence of *D. immitis* infection of 15.3%, based on both parasitological and antigen detection methods.

Data analysis showed only a moderate level of agreement between the tests (Landis & Koch, 1977). Among the 308 samples tested, 24 (7.8%) were positive for *D. immitis* and 261 (84.7%) were negative by both methods (Kappa = 0.634, $P < 0.001$). These findings indicate that at least two distinct methods should be used to screen for canine dirofilariosis, as suggested by Genchi et al., (2007).

As far as we know, this is the first report of the presence of *W. pipientis* in Portuguese dogs infected with *D. immitis*. *Wolbachia pipientis* DNA was detected in the blood of approximately half of all microfilaremic dogs. This prevalence is lower than the 100% found in Brazil (Rossi et al., 2010), but higher than the 30.6% reported in Spain (Tabar et al., 2013). However, the detection limit for the PCR conditions used here, 14 pg of DNA from adult female *D. immitis*, is almost 6 times lower than that reported by Rossi et al., (2010) for a PCR product half the size of the fragment produced in this study. Samples with low microfilaraemia were positive here by PCR even to dilutions of up to 1:100, so, it is unlikely that the low percentage of positivity by PCR in microfilaremic dogs is due to a small number of microfilariae. The presence of PCR inhibitors is unlikely, given that DNA dilutions were still negative, as is the possibility that DNA was degraded, since all samples were processed similarly. Thus, other factors related to *W. pipientis* and/or canine sampling may account for our findings; it is possible that microfilariae had a very low concentration of bacterial DNA, as a result of any antibiotic treatment administered to those dogs, or that there were DNA sequence variants at a primer site, undetectable by the PCR used. In any case, these results indicate that detection of *W. pipientis* in the blood by this PCR could not be used as an alternative diagnostic method for *Dirofilaria* infection, as proposed by Lee, Lee, Choi and Hyun (2008).

These findings reinforce the observations that microfilariae may represent an important source of *W. pipientis* dissemination in the host (Taylor, Voronin, Kelly, Johnston & Ford, 2013). However, a widespread survey of the prevalence of *W. pipientis* indifferent *D. immitis* populations is needed, as suggested by Tabar et al., (2013), in order to understand why in some cases, it is not possible to detect *W. pipientis* in dogs with dirofilariosis, as observed in our and another recent study (Tabar et al., 2013). In addition, further research on *Wolbachia–Dirofilaria* interactions, not only in individual adult worms, but with particular emphasis on immature forms (microfilariae), could provide additional information about the disease pathology and unravel new targets for specific diagnosis or treatment schedules. Furthermore, these studies will drive further national and regional surveys, leading to a better knowledge of the importance

of the *D. immitis*/*W. pipientis* complex on the epidemiology and control of canine heartworm disease.

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CHAPTER 5

Assessment of the transmission risk of *Dirofilaria* spp. in Portugal

Chapter 5, Scientific publication 1

Transmission risk of dirofilariosis in Portugal

Ana Margarida Alho[1]*, Telmo Nunes[1], Laura Rinaldi[2], José Meireles[1], Silvana Belo[3], Peter Deplazes[4] & Luís Madeira de Carvalho[1]. Proceedings of the *1st Conference on Neglected Vectors and Vector-Borne Diseases*, Cluj-Napoca, Romania, 8-11 April 2014. *Parasites & Vectors*, 7 (Suppl 1): O16. [**abstract**]

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* The author performed the data analysis process and co-produced the abstract.

Abstract

Dirofilariosis is an important and potential fatal vector-borne canine disease, endemic in Portugal as in other Mediterranean countries, particularly due to its geographic and climatic conditions. Despite improved diagnostic methods and effective preventives, numbers of *Dirofilaria* spp. infections continue to increase. Forty-one species of mosquitoes have been detected in Portugal mainland although only *Culex theileri* was found naturally infected with *Dirofilaria* larvae. Therefore, a study was addressed to assess *Dirofilaria* transmission risk in Portugal based on air temperatures, estimating the potential days with temperature values compatible with the transmission of *Dirofilaria* larvae between mosquito and reservoirs. A degree-days model based on *Dirofilaria* Development Units (DDUs) was used, considering minimum and maximum daily temperatures registered in five Portuguese meteorological stations, obtained in the platform wunderground.com. Preconditions for the model were: a threshold temperature of 14ºC below which *Dirofilaria* development will not proceed in mosquitoes; 130 cumulative DDUs for larvae to reach infectivity; and a maximum life expectancy of 30 days for mosquito vectors.

DDU was evaluated in three areas of Portugal mainland - Porto (North, 41°9'0"N/8°37'0"W), Lisbon (Center, 38°43'0"N/9°8'0"W) and Faro (South, 37°1'0"N/7°56'0"W) as well as two different Portuguese islands in the Atlantic Ocean – São Miguel, Azores (37°44'0"N/25°40'0"W) and Madeira (32°38' 0"N/16°54'0"W), over the period from 2003 to 2013. The results show that the highest number of potential days with suitable conditions for *Dirofilaria* transmission was registered in Madeira with an average of 209.9 days/year, followed by Faro 175.2 days/year, Lisbon 163.5 days/year, Azores 140 days/year and Porto 117.2 days/year. The year 2006 was the one with a maximum number of potential transmission days (179.8) across the selected stations. During the last decade, 130 DDUs were inclusively registered from June to November in Porto and Azores Island, from April to November in Lisbon and Faro, and an uncommonly extended period from April to January in Madeira Island. An average *Dirofilaria* seasonal risk period ranged from a minimum of 5 months/year in Porto, 5.6 months/year in Azores, 6.4 months/year in Lisboa, 6.9 months/year in Faro and 8 months/year in Madeira.

These results are in accordance with the existing prevalence data and reinforce the value of geospatial tools, mapping the risk and helping to monitor and forecast future epidemiological trends, ensuring a continued surveillance and a “One Medicine–One Health” integrated approach.

CHAPTER 6

A new mechanical therapeutic approach for removal of *Dirofilaria immitis*

Chapter 6, Scientific publication 1

A homemade snare: an alternative method for mechanical removal of *Dirofilaria immitis* in dogs

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Abstract

Canine dirofilariosis is a life-threatening parasitic disease that is increasingly reported worldwide. Once diagnosed the main treatment goals are to improve the animal's clinical condition and to eliminate all life stages of the parasite with minimal posttreatment side effects. This can be achieved through mechanical, surgical, or chemotherapeutical approaches. Currently manual extraction is the preferred method to remove adult heartworms due to its diminished invasiveness, reduced damage to the vascular endothelium, and shortened anaesthesia duration. However, it remains an expensive technique that can be highly traumatic. To address this issue, a non-traumatic homemade catheter-guided snare was developed for heartworm removal by adapting and folding a 0.014-inch coronary wire (BMW, Abbott Vascular). Transvenous heartworm extraction was performed on a dog severely infected with adult heartworms by inserting the modified snare into a 6-F Judkins right coronary guiding catheter BMW (Cordis) and advancing it into the right ventricle under fluoroscopic guidance. Fifteen adult specimens of *Dirofilaria immitis* were successfully extracted from the pulmonary artery and right ventricle without complications. To assure the death of both larvae and adults, postoperative treatment was successfully managed using ivermectin, doxycycline, and melarsomine, with no recurrence after surgery.

1. Introduction

Canine dirofilariosis is a severe canine vector-borne disease with potentially fatal consequences. It is widely distributed throughout the world, with an increasing incidence in previously nonendemic areas (Morchón, Carretón, González-Miguel & I. Mellado-Hernández, 2012; Simón et al., 2012). The main treatment goals are to improve the animal's clinical condition and to eliminate all forms of the parasite (microfilariae, larval stages, juveniles, and adults) with minimal complications. This can be achieved pharmacologically by combining melarsomine dihydrochloride, macrocyclic lactones, and doxycycline (Nelson, McCall & Carithers, 2014). However, this approach can lead to several complications and adverse effects including pulmonary thromboembolism due to the worm death and anaphylactic shock secondary to the sudden death of high microfilariae counts (Atkins, 2010). For this reason, either mechanical or surgical heartworm removal is generally preferred as a means to eliminate as many adult worms as possible before pharmacological treatment is initiated. Manual extraction is the preferred method due to its diminished invasiveness, reduced damage to the vascular endothelium, and shortened anaesthesia duration (Atkins, 2010; Bové & Gordon, 2010). However, it remains an expensive technique out of the reach of many owners. Additionally, some of the available devices are also traumatic. To address these issues a non-traumatic intravascular snare was developed by adapting an economical coronary wire (commonly used in human patients) to attempt heartworm removal.

2. Materials and methods

2.1. Case presentation

A senior unneutered mixed-breed male dog (body weight: 6.1 kg) was presented to the Small Animal Teaching Hospital of the Faculty of Veterinary Medicine, ULisboa, with a history of severe cough, weakness, dyspnoea, exercise intolerance, and syncope. The owner reported a recent episode of hind limb weakness and temporary loss of balance which lasted for approximately 40 seconds. The dog was adopted from a shelter one month prior to presentation and his age was unknown (10 years old approximately). During the intervening period between adoption and presentation at the hospital no prophylactic treatment was initiated by the owner. On physical examination, the dog had normal weight and was alert and responsive, but it was tachypnoeic and slightly dyspnoeic. Mucous membranes were pink with a capillary refill time of less than two seconds. Thoracic auscultation revealed an increased respiratory effort associated with mild crackles. A loud systolic regurgitant heart murmur (grade III approximately) was audible on the right side of the thorax, more significantly over the tricuspid

valve and near the right side of the heart apex. The remainder of the physical examination was unremarkable.

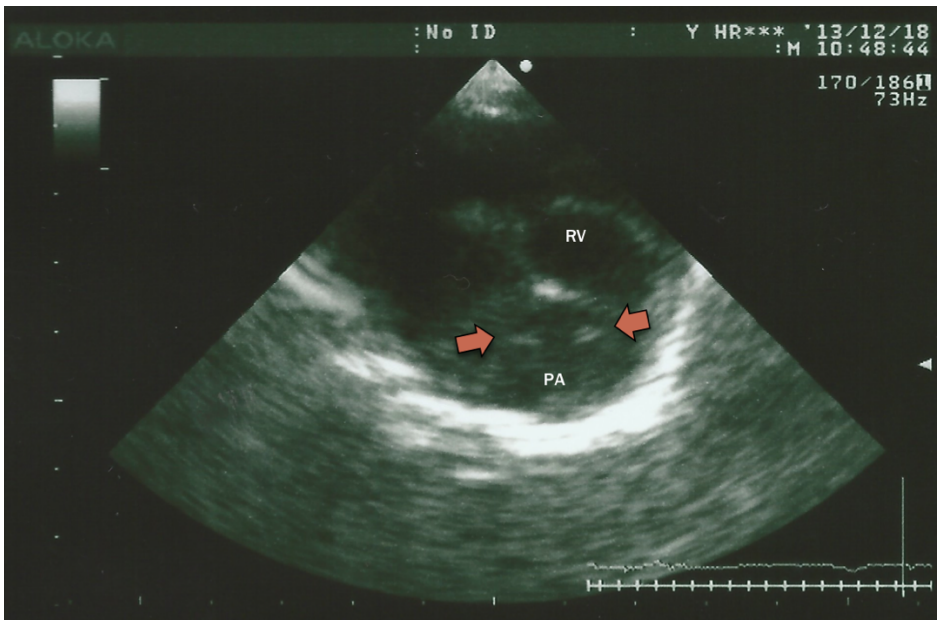
2.2. Diagnostic methods

Blood was collected from the cephalic vein and direct smears were performed. Under light microscopy, several microfilariae were observed. These were identified as *Dirofilaria immitis* using Knott modified test based on their morphometric characteristics (Magnis et al., 2013). The commercial WITNESS® *Dirofilaria* kit (Synbiotics Corp., Europe) was also used, supporting the previous diagnosis.

A complete blood count was performed [white blood cell count, 11.3 $x10^3/\mu L$ (6–17 $x10^3/\mu L$); red blood cell count, 6.2 $x10^6/\mu L$ (5.5–8.5 $x10^6/\mu L$); platelet count, 347 $x10^3/\mu L$ (200–500 $x10^3/\mu L$); haemoglobin, 14.1 g/dL (12–18 g/dL); haematocrit, 46.8% (37–55%); eosinophils, 1.4 $x10^3/\mu L$ (0.1–1.3 $x10^3/\mu L$)], revealing a mild eosinophilia. Routine serum biochemistry profile was also performed [glucose, 107 mg/dL (60–125mg/dL); total protein, 9.0 g/dL (5.1–7.8 g/dL); creatinine, 0.7mg/dL (0.4–1.8mg/dL); alkaline phosphatase, 116 μl/L (10–150 μl/L)]. Prerenal azotaemia [blood urea nitrogen, 52.3 mg/dL (7–27 mg/dL)] and moderately increased hepatic enzymes, alanine transaminase (ALT) [240 μl/L (5–60 μl/L)] and aspartate transaminase (AST) [96.6 μl/L (5–55 μl/L)], were found, possibly explained by passive liver congestion due to right cardiac overload.

To assess the severity of heartworm cardiopulmonary disease, lateral and ventrodorsal radiographic projections of the thorax were made at full inspiration, revealing slight dilation of the right ventricle and bulging of the pulmonary arteries. Vertebral heart score was 9.8 (8.7–10.7) and there was no evidence of lung inflammation in the areas surrounding the pulmonary arteries. Further transthoracic echocardiography revealed the presence of linear, mobile, parallel hyperechoic structures (short parallel-sided images with the appearance of “equal signs”) within the right ventricle outflow tract and main pulmonary artery, consistent with the presence of heartworms (Fig. 1). Spectral Doppler echocardiography showed mild tricuspid regurgitation (velocity of 2.3 meters per second). Additionally, slight dilation of the right ventricle was noticed without increase of the pulmonary flow velocity or abnormal tricuspid relation between E wave and A wave [E:A ratio]. No heartworms were visualized within the tricuspid orifice or posterior vena cava, excluding the diagnosis of caval syndrome.

Figure 1 - An echocardiographic image of the right ventricle (RV) and pulmonary artery (PA), in a short axis view, right parasternal section, in a right lateral decubitus. Note the presence of linear, parallel hyperechoic structures corresponding to adult worms (arrows) within the pulmonary artery.



Considering the clinical signs exhibited by the dog (coughing, exercise intolerance, weakness, dyspnoea, and syncope), the abnormal findings on the thoracic radiography, and the visualization of hyperechoic structures consistent with parasites within the right ventricle and pulmonary artery, it was concluded that the dog was severely infected with heartworms (Nelson et al., 2014) and was at high risk for thromboembolic complications. As overall survival is significantly improved in animals that undertake mechanical heartworm removal (prior to the adulticide therapy) (Nelson et al., 2014), and as the echocardiography showed worms in accessible locations to be percutaneously removed, heartworm removal procedure was proposed using a homemade snare. Owner's informed consent was given and heartworm removal was scheduled.

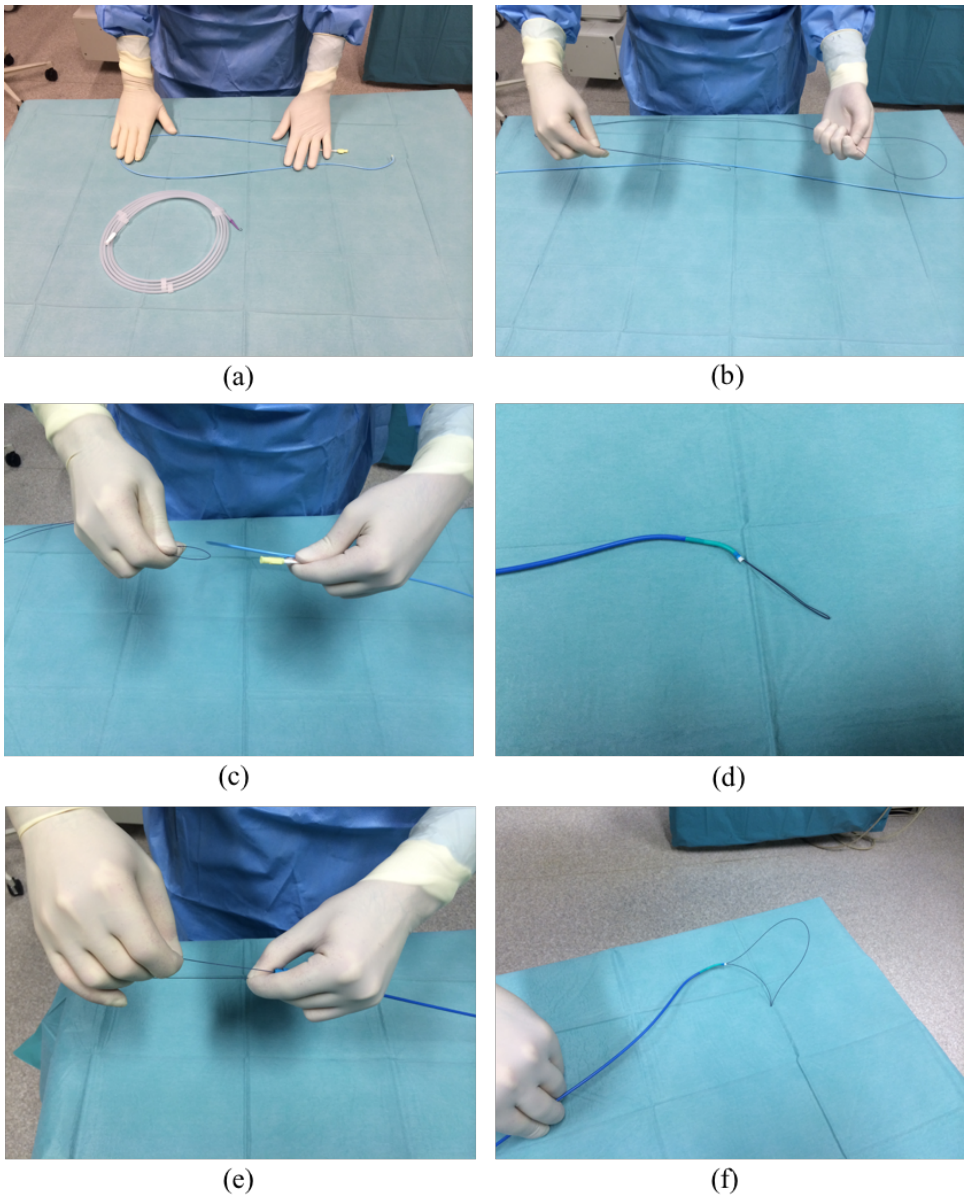
2.3. Transvenous heartworm extraction procedure

Two weeks prior to surgery the dog was stabilized with furosemide (1mg/kg, per os [PO], twice a day [BID]) and enalapril (0.5mg/kg, PO, once a day [SID]) to minimize cardiac overload. Also doxycycline (10mg/kg, PO, BID) was prescribed at the same time. In order to reduce the thromboembolic risk associated with heart catheterization and adult worm death, the dog was also started on prednisolone (0.5mg/kg, PO, BID) and cetirizine (1mg/kg, PO, SID) one week prior to the procedure.

On the day of the procedure, the dog was premedicated with heparin (100 U/kg, subcutaneously [SC]) and an association of amoxicillin and clavulanic acid (20mg/kg, BID, intramuscularly [IM]). Anaesthesia was induced with propofol (4mg/kg, intravenously [IV]) and maintained with isoflurane (2–2.5% concentration) after tracheal intubation. The dog was kept in left lateral recumbence and the right side of the cervical region was prepared. Venous puncture was performed using the Seldinger technique and a 6-F plastic sheath was introduced via the right external jugular vein.

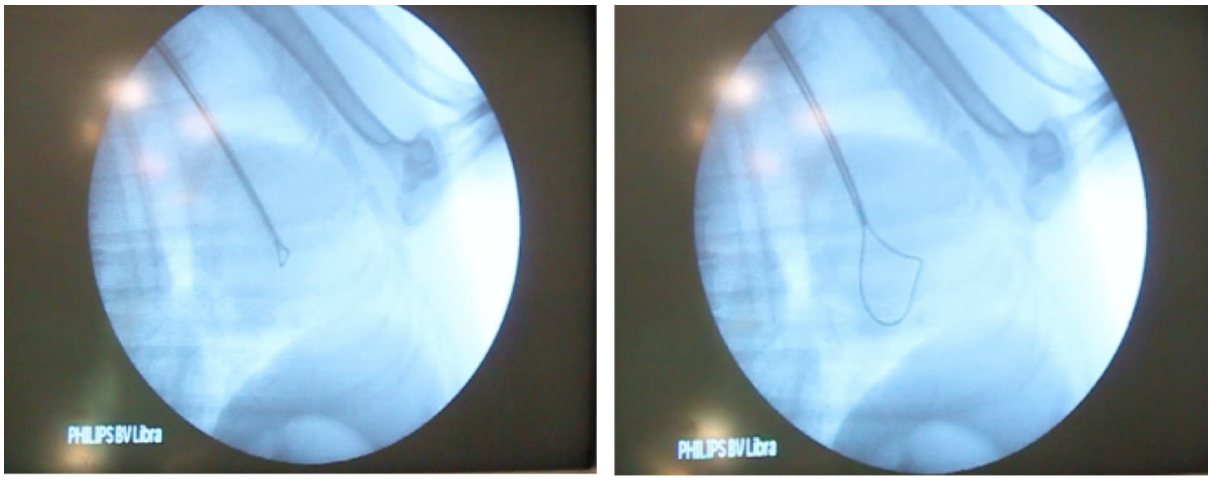
Anticoagulation was enhanced with intravenous heparin (100 U/kg). Under fluoroscopic guidance, a 6-F Judkins right coronary guiding catheter BMW (Cordis) was introduced and moved towards the cranial vena cava, right atrium, and right ventricle. A homemade snare was created by folding a 0.014-inch coronary wire (Boston Scientific) (Fig. 2).

Figure 2 - Mechanical heartworm removal device used during the procedure. (a) A snare introducer, a 6-F plastic sheath, inserted via the right external jugular vein. (b) A specific carrier, a 6-F Judkins right coronary guiding catheter BMW (Cordis). (c), (d), and (e) A 0.014-inch coronary wire (Boston Scientific) that was folded and pushed through the coronary guiding catheter. (f) Final aspect of the homemade snare.



This device was subsequently inserted into the guiding catheter keeping both distal parts exteriorized. The operator fixed one of the wire extremities with one hand and moved the other end forward, adapting the size and shape of the loop according to the number and location of the worms (Fig. 3). To retract the worms through the catheter, both extremities of the wire were gently withdrawn at the same time.

Figure 3 - Heartworm surgical extraction under fluoroscopy guidance. (a) A 6-F Judkins guiding catheter BMW (Cordis) and the loop wire, placed at the right ventricle. (b) Increasing the size of the loop wire in order to snare the heartworms, followed by gentle retraction of the snare.



(a) (b)

Since navigation into the pulmonary artery was difficult with the 8-F guiding catheter, it was downsized to a 6-F model. For this reason, a smaller device was created using 0.014-inch coronary wire (BMW, Abbott Vascular), which was folded using the same method described previously. This homemade snare was then used to pull out the remaining heartworms through the sheath.

3. Results

In total, fifteen adult specimens of *Dirofilaria immitis* were caught and gently retracted through the catheter from the right ventricle and proximal portion of the pulmonary artery (Fig. 4).

Figure 4 - Retracted worms: the 15 specimens of *Dirofilaria immitis* extracted with the homemade snare from the right side of the heart and pulmonary artery.

Considering the risks of cardiac arrest and potential heart and vascular lesions due to continued catheter manipulation as well as the prolonged duration of the anaesthesia, the catheter was retracted and no further attempts were made. Haemostasis was achieved with manual compression and the dog was sent to the intensive care unit after the procedure was completed. Recovery occurred without complications and the dog was discharged after careful evaluation with amoxicillin and clavulanic acid (20mg/kg, BID, PO) and with instructions for the owner to restrict exercise.

A postoperative reevaluation was scheduled seven days after the procedure. Once the dog was recovering well, treatment with ivermectin (10 μg/kg, PO) was initiated to prevent potential residual infection. Doxycycline (10mg/kg, PO, for 28 days, BID) was also restarted. The first melarsomine injection was performed 60 days after surgery (2.5mg/kg, IM). The second and third consecutive treatments were performed 90 and 91 days after surgery, as recommended by the American Heartworm Society (Nelson et al., 2014). Exercise restriction was imposed during the entire treatment regimen.

Three months after surgery, the dog was reevaluated. Clinical signs relating to the presence of heartworms were resolved and no murmurs were auscultated. The owner reported that the dog had a good appetite and energy levels but still coughed occasionally. Routine heartworm prevention on a monthly basis was recommended. Eight months after surgery, the dog was very alert and active and no coughing was reported. An additional commercial antigen, WITNESS *Dirofilaria* kit, was performed, testing negative for *D. immitis* infection.

4. Discussion and conclusion

In order to offer an affordable and safe treatment to every owner, in cases where mechanical heartworm removal is the most appropriate treatment, a catheter-guided technique using a homemade snare for adult heartworms retrieval was developed.

In general, mechanical extraction is a far less invasive and painful method when compared to cardiothoracic surgery, allowing a faster recovery and reducing the risk of infection. The snare is a safer technique when compared with forceps or the horsehair brush, since it minimizes accidental intracardiac and vascular damage, frequently associated with blind grasping (Small et al., 2008). The snare is also advantageous in comparison with the basket retrieval device, since the operator can control the degree of closure of the snare and thus reduce the risk of traumatizing or breaking the ensnared worms (Small et al., 2008). The snare's loop can also be manipulated to adopt the size, shape, or angle intended by the practitioner, increasing the likelihood of worm retrieval. In addition, since venotomy is not required to access the jugular vein, surgical closure is not necessary and the subsequent bleeding associated with catheter insertion is practically insignificant. The snare also appears to be more effective over previously described heartworm extraction methods, namely, Ishihara and flexible alligator forceps, whose size only permits their introduction into the right atrium and proximal portion of the right ventricle and not through the tricuspid and pulmonic valves (Small et al., 2008). Furthermore, this homemade snare is less expensive than the specific snare usually employed for this task, since it only requires a sheath, a coronary guiding catheter, and a common coronary wire.

Despite the abovementioned advantages, general anaesthesia, fluoroscopic guidance, subsequent chemotherapy, and a skilled practitioner are still required (Small et al., 2008; Atkins, 2010). Besides, the potential risk for cardiac arrest and ventricular arrhythmias caused by snare manipulation in the right ventricle or even the risk of transecting an adult heartworm still remains.

Without direct visualization of the worms, the success of percutaneous heartworm extraction will always rely upon the operator's ability to ensnare the worms, which is dependent on their anatomical location and burden and the size of the parasites. To accomplish a more efficacious heartworm extraction, care must be taken to move one of the snare tips while the other is maintained in a fixed position, in order to achieve the necessary loop size.

Scant data is currently available in the literature regarding transvenous procedures for adult heartworm retrieval in companion animals. The most common reported devices used are Ishihara forceps, Jones forceps, the horsehair brush, tripod forceps (Yoon, Choi, Lee & C. Hyun, 2013), basket forceps (Yoon, Han & Hyun, 2011; Yoon et al., 2013), alligator forceps (Sasaki, Kitagawa, Ishihara & Masegi, 1990; Atwell & Litster, 2002; Arita, Yamane &

Takemura, 2003; Yoon et al., 2005), endoscopic grasping forceps, flexible three wires nail tipped forceps (Lee, Moon & Hyun, 2008), and the gooseneck snare (Small et al., 2008). More sophisticated commercial snares, which include the nitinol gooseneck snare, have total and reproducible memory allowing the loop to return to a specific shape and diameter, a considerable advantage over the homemade snare. But, evidently, these are more expensive and thus are not a viable alternative for low-cost surgery (Mullins, 2006).

Further surgical transvenous interventions need to be done to validate and improve the efficiency of this technique. Nevertheless, we believe that the possible cost reductions and less traumatic damage induced by this snare, when compared to existing alternatives, will allow adult heartworm extraction to be more affordable and consequently widespread, thereby promoting the treatment of a larger number of animals, enhancing a specific chemotherapy with higher safety.

Abbreviations

ALT: Alanine transaminase; AST: Aspartate transaminase; BID: Twice a day; E:A ratio: Relation between E wave and A wave; IM: Intramuscularly; IV: Intravenously; PO: Per os; SC: Subcutaneously; SID: Once a day.

Ethical Approval

All technical procedures were in accordance with National (DL 276/2001 and DL 314/2003) and European Legislation regarding animal welfare and met the International Guiding Principles for Biomedical Research Involving Animals by the Council for the International Organizations of Medical Sciences.

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CHAPTER 7

Parasite control practices and public perceptions of pet owners in Portugal

Chapter 7, Scientific publication 1

Parasite control practices in companion animals: a survey of dog and cat owners

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* The author conceived the study, helped in the data analysis and co-produced the manuscript.

Abstract

Drugs used in the control of internal and external parasites in companion animals play a crucial role in Animal and Public Health. To ensure continuing protection, these drugs should be administered regularly and in intervals, as suggested by the manufacturers. To assess parasite control practices and other related factors, including the degree of public awareness on the topic, 312 dog and cat owners were surveyed while attending the Small Animal Hospital, Faculty of Veterinary Medicine, Lisbon University. Results showed that 89.7% of the dogs were currently being treated with endoparasitic drugs. Of these,74.3% were dewormed every four months or longer and merely 11.8% with the recommended treatment regimen (minimum quarterly). In cats, 63.6% were being treated with endoparasitic drugs and 85.7% of these were irregularly dewormed every four months or longer and merely 5.5% with the recommended treatment regimen (minimum quarterly). Combinations of praziquantel, pyrantel embonate and febantel were the most commonly used drugs in dogs, whereas macrocyclic lactones were more frequently used in cats. Regarding external parasitic control, 92.2% of the dogs were being treated, 50.5% of these at monthly intervals (all-year round or seasonally). The most common ectoparasitic drug formulation used on dogs was the spot-on imidacloprid + permethrin (89%). Only 28.4% of the dogs were uninterruptedly protected throughout the year from the main canine vector borne diseases transmitted by fleas, ticks, sandflies and mosquitoes. Merely 63.6% of the cats were being controlled with ectoparasitic drugs, most at infrequent drug intervals and imidacloprid was the most frequently used drug on cats (44.4%). Additionally, 85% of the respondents had never heard of the word “zoonosis” and 37% of them did not collect their dog’s faeces in all public places. Scabies, toxoplasmosis and leishmaniasis were the most frequent parasitic diseases identified by the public in this survey. Although the majority of pet owners give antiparasitic drugs, our results show that most of them do not follow the manufacturers recommendations, deworming at irregular and consequently ineffective intervals. Therefore, it is of utmost importance for the veterinarians to educate pet owners regarding parasite cycles, methods of prevention and transmission mechanisms, as well as to follow the drug recommendations, in order, respectively, to increase their awareness and thereby improve the effectiveness of the available control measures.

Keywords: Dog, Cat, Prophylaxis, Parasite control, Zoonosis, One health.

1. Introduction

Despite advances regarding prophylaxis and treatment of parasitic diseases, parasites are still responsible for significant morbidity and mortality in companion animals. Furthermore, their zoonotic potential frequently presents an environmental and Public Health menace (Page, 2008; Bowman, 2009).

The term endoparasites, apart from "traditional" intestinal worms, also covers other (extra-intestinal) parasites such as *Dirofilaria immitis, Aelurostrongylus abstrusus, Angiostrongylus vasorum* and other vector borne agents (protozoa), namely *Leishmania infantum* and *Babesia* spp. Recent attention from the scientific community to extra-intestinal parasites has caused the misconception that intestinal parasites in dogs and cats are no longer important, mainly because the routine use of certain anthelmintics (AH) is believed to have reduced their diffusion and impact on animal health and welfare (Traversa, 2012). In fact, intestinal protozoan infections caused by *Giardia* spp., *Cryptosporidium* spp. (both zoonotic) and *Cystoisospora* spp. (all endoparasites) are affecting more and more dogs and cats; in Portugal, studies have shown that *Giardia* spp., *Cystoisospora* spp., *Toxocara* spp., *Toxascaris leonina*, *Ancylostoma* spp., *Trichuris* spp. and *Dipylidium caninum* were the most prevalent endoparasites in small animals (Duarte et al., 2010; Ferreira et al., 2011; Lebre, 2011; Neves, Lobo, Brilhante Simões & Cardoso, 2013). Most of these studies show a higher prevalence of protozoan infections (especially *Giardia* spp.) than helminth infections, which may be due to the fact that most of the endoparasiticides used worldwide are AH, and are therefore ineffective against this sort of infection (Little et al., 2009).

Several studies conducted across the country revealed the presence of parasites of zoonotic concern, namely Ascarididae and Ancylostomatidae (Crespo et al., 2006), especially *Toxocara* spp., detected in 80% of public parks (Otero et al., 2013). This is also the reason why the European Scientific Counsel Companion Animal Parasites guidelines (ESCCAP, 2010) expresses the need to implement environmental control measures (namely dog faeces removal) along with effective worm control in dogs and cats. Worm control with appropriate AH is recommended on at least a quarterly basis, especially when the pet owner does not perform routine coprology tests, which are also suggested as an alternative to repeated treatments (ESCCAP, 2010). ESCCAP guidelines also mention what Sager et al. (2005) demonstrated: an increase of treatment frequency effectively reduces the occurrence of infected animals; deworming four times/year does not necessarily eliminate patent infections. However, a monthly worm treatment can largely prevent patent infections, as it takes into account the cycle of the parasites. It is important to note that repetitive AH use has been associated with the

emergence of AH parasite resistance in small animals, mainly with pyrantel (Kopp, Coleman, Traub, McCarthy & Kotze, 2009) and macrocyclic lactones (Bowman, 2012).

In an ever-changing world, various factors may be potentiating an increase in exposure to old and new parasitic agents, with some even re-emerging. It is known that climatic factors are transforming the epidemiology of certain parasites, but other factors are also involved, such as urbanization and deforestation, demographic and political changes, making the spread of ectoparasites and their pathogens a no-boundaries global event (Colwell, Dantas-Torres & Otranto, 2011).

In Portugal, canine vector-borne diseases (CVBD) represent a growing concern amongst veterinarians and parasitologists. Recently, a national serological study in healthy dogs revealed that 14% were positive for one or more of the following agents: *D. immitis, Ehrlichia canis, Borrelia burgdorferi* sensu lato*, Anaplasma* spp. and *L. infantum* (Cardoso, Mendão & Madeira de Carvalho, 2012). Other national data showed an apparent prevalence for *L. infantum* in dogs ranging from 0.9% to 16.2%, with the highest prevalence in the interior regions (Cortes et al., 2012). Canine *L. infantum* is endemic in Portugal and lately the scientific community has been studying the role of cats as an alternative reservoir rather than an accidental host (Maia et al., 2010). This lends credence to the view that an effective ectoparasite control approach is of utmost importance in dogs and cats. ESCCAP control guidelines (2012a,b) recommend that animals with outdoor access must be treated with insecticides-acaricides at appropriate treatment intervals (generally monthly, according to product label recommendations) for effective prevention. They also state that acaricide treatment should continue throughout the year in warmer areas and that seasonal insecticide repellent treatment must take place to prevent mosquito and sandfly related diseases. Some authors are ultimately debating the "seasonality paradigm" of CVBD, warning that the occurrence of CVBDs should no longer be considered a seasonal phenomenon (Otranto, Dantas-Torres & Breitschwerdt, 2009).

Little is known about which parasitic control practices are in place and how pet owners implement them on their dogs and cats. Furthermore, the level of public knowledge about parasites and parasitic diseases, as well as, the safety measures partaken by the owners in public places, needs to be evaluated, to encourage general awareness about these topics. The present study is intended to answer those questions in a survey performed among pet owners in order to assess the way dogs and cats are being protected from parasites in Portugal, especially those attending a reference animal hospital in the Lisbon area.

2. Material and methods

2.1 Study design, topics and procedures

The authors conducted this survey as an oral personal interview. The interviewees included dog and/or cat owners who attended our Small Animal Hospital, Faculty of Veterinary Medicine, University of Lisbon, from January to April 2013. In order to assess and characterize the differential antiparasitic control strategies, priority was given to owners that visited the hospital for a second opinion/specialist appointment from other clinics.

Sixty sample interviews were conducted to test and assess the viability of different survey formats and questions (written and multiple-choice answers). The final format was a multiple-choice based interview that took approximately 7 to 10 minutes to complete, followed by a small period to clarify owner's doubts on the subject. The study included groups of questions on the following topics: a) characterization of the use of endo- and ectoparasiticides on the animal; b) respondent's knowledge concerning parasites and parasitic diseases; c) animal characterization along with questions specially assigned for dog owners, about dog faeces removal in public places; and d) interviewees characterization. The questions were formulated in order to be easy to understand and in a way to enable the owners to respond freely, without feeling constrained about any compromising answers that could lead them to give what they deem to be the "correct" answer rather than the truth.

ESCCAP guidelines (ESCCAP, 2010; 2012a,b) were considered to determine the adequate preventive antiparasitic control strategy, which was at least quarterly for worm control (without faecal analysis) and monthly for ectoparasite control, for animals with regular outdoor access. In order to assess the number of dogs and cats that were adequately and continuously protected (considering ESCCAP guidelines), three parameters were defined: a) owner compliance with the recommended treatment schedule; b) treatment given at least quarterly; c) at least one treatment in the last three months.

Owners were also asked a series of questions intended to assess their knowledge on parasites as a potential cause of infection and disease, and also about their perception of possible ways their pets may acquire endoparasitic diseases.

2.2 Statistical analysis

Statistical analysis was performed using R program 3.0.0 (The R Foundation for Statistical Computing, 2013). Summary statistics for continuous variables were expressed as mean and standard deviation (±), median, 25^{th}, 75^{th} percentiles (25, 75P) and interquartile range (IQR). Nevertheless, almost all variables were of categorical data, expressed as numerator,

denominator and/or per cent. Association and comparison of categorical proportion outcomes between respondents were done with a two-sided X^2 test and two-sided Fisher's exact test, considering $p<0.05$ as a statistical significance level. Confidence limits were calculated as described by Reiczigel, Földi and Ózsvári (2010).

3. Results

A total of 312 interviews were conducted, 243 (77.9%) were dog owners and 69 (22.1%) cat owners. Interviewee employment status was as follows: 56.09% employed; 19.23% retired; 15.38% unemployed; and 9.29% students.

Considering the different parasitic control practices relating to adult and young animals, the sample was divided in two main groups, and then subdivided according to species: adult animals (considered ≥ 1-year-old): 204 dogs (65.4%) and 55 cats (17.6%); and young animals (considered ≤ 1 y.o.): 39 puppies (12.5%) and 14 kittens (4.5%).

Approximately 65% of the dogs were purebred (Labrador Retriever, Poodle and Cocker Spaniel were the most represented breeds), with a median age of 8 y.o. (IQR: 1.18). The majority of the cats (89%) were European shorthairs, with a median age of 8 y.o. (IQR: 1.22). Concerning outdoor access allowance, 90% (62/69) of the surveyed cats were exclusively indoor whereas 80% of the dogs (195/243) had regular outdoor access ($X^2_{df:1}=121.4$; $P<0.001$) and frequent contact with other outdoor animals (mostly dogs). Dogs were walked most frequently in the morning and evening. Of these, 57% were walked at dusk.

3.1 Parasite control practices: endoparasiticides

Out of the 86.5% interviewees (n=270/312) who mentioned the use of AH drugs in their pets, 96.7% did so as a prophylactic measure; 2.6% for suspected parasitic infection; and 0.7% as treatment for a previously diagnosed infection.

3.1.1 Dogs

At the time of the study, out of the total number of adult dogs surveyed (n=204), 89.7% (n=183) were being treated with endoparasitic drugs (Table 1). Despite these findings, 42.4% of the dog owners admitted forgetfulness to re-administer AH drugs thereby, not complying with the scheduled treatment. Applying the ESCCAP parameters mentioned before to assess the number of animals that were adequately and continuously protected, a mere 11.8% (n=24/204) fulfilled all three requirements; out of these, the majority were purebred (83.3%; $P=0.03$), with an

average age of 6 y.o. (±3.9) and belonged to active working owners (75%; $X^2_{df:1}$=4.6; P=0.03) with a median age of 37 y.o. (25, 75P: 31, 47). Conversely, only 59% of the dogs that were dewormed every four-months or longer were purebred. They were also slightly older (average age of 8 y.o. ±4.2) and pertained to more mature owners (median=49; 25, 75P: 34, 60). The small number of untreated dogs (13/204), were also older than the previous groups (75% ranging from 9 to 18 y.o.).

Table 1 – Endoparasite control practices performed in adult dogs and cats**.**

	Dogs		Cats		*P* value
	n	(%)	n	(%)	
Total number of adult animals analysed	204		55		
Treated with AH	**183**	**(89.7)**	**35**	**(63.6)**	**<0.001**
-Treated with AH at least every 3-months	**47**	**(25.7)**	**5**	**(14.3)**	
Every month	11	(6)	-	-	
Every 2-months	5	(2.7)	-	-	
Every 3-months	31	(16.9)	5	(14.3)	
-Treated with AH every 4-months or longer	**136**	**(74.3)**	**30**	**(85.7)**	
Every 4-months	41	(22.4)	4	(11.4)	
Every 6-months	56	(30.6)	13	(37.1)	
Once a year	20	(10.9)	8	(22.9)	
Other (irregular intervals/doesn't know)	19	(10.4)	5	(14.3)	
Treated with AH in the previous 3 months	**103**	**(50.5)**	**19**	**(34.6)**	**0.08**
Treated continuously* with AH	**24**	**(11.8)**	**3**	**(5.5)**	

Note: AH – anthelmintic; *In accordance with guidelines and considering the three parameters previously defined: a) owner compliance with the recommended treatment schedule; b) treatment given at least quarterly; c) at least one treatment in the last three months**.** *P* values refer to statistical differences between dogs and cats.

We also found that 97.4% (n=38) of the puppies were dewormed and that 83% of their owners rigorously followed a scheduled programme, mostly on fortnightly or monthly basis. A significant statistical difference was found in deworming compliance between puppy and adult dog owners (P=0.001). Another significant statistical difference was found concerning deworming guidance: puppy owners usually deworm following veterinarian recommendation, whereby adult dog owners were found to do so at their own initiative, attributing deworming as a "common-sense" practice ($X^2_{df:1}$=16.7; P<0.001).

The most commonly used AH drugs in adult dogs (56.9%) was the combination of praziquantel-pyrantel embonate with a third molecule (febantel, oxantel or fenbendazole). Macrocyclic lactones were only used 13.1% of the time (Table 2). One owner used homeopathic products for deworming control. Almost all puppy owners referred to using a praziquantel-mebendazole oral gel paste as the AH formulation of choice on their pets.

Table 2 - Endoparasiticides used in adult dogs and cats**.**

Adult Dogs	**%** (95%CL)	**Adult Cats**	**%** (95%CL)
PRQ+Pyrantel+ Febantel/Oxantel/FBZ	56.93 (48.5-65.1)	Macrocyclic lactone (comb.)	72.73 (50-87.4)
Macrocyclic lactone (comb.)	13.14 (8.1-19.9)	PRQ+Pyrantel/Pyrantel	18.18 (6.5-38.9)
PRQ+Emodepside	8.76 (4.8-14.7)	Other (comb.)	9.09 (1.6-28.7)
PRQ	5.84 (2.6-11.1)		
FBZ /+PRQ	5.84 (2.6-11.1)		
Epsiprantel+Pyrantel	4.38 (1.9-9.2)		
Niclosamide+OxiBZ	2.19 (0.6-6.3)		
Pyrantel	1.46 (0.3-5.2)		
Nitroscanate	1.46 (0.3-5.2)		

PRQ: Praziquantel; FBZ: Fenbendazole; OxiBZ: Oxibendazole; comb.: includes molecule combinations

3.1.2 Cats

Regarding the application of endoparasitic drugs in cats, more than one third of the adults, i.e. 36.4% (20/55), were not dewormed at all. Out of the dewormed cats, 85.7% were treated every 4-months or longer (Table 1); 57.1% of the respondents mentioned that they frequently did not comply with the scheduled treatment. Considering the deworming frequency cited in literature (ESCCAP guidelines) and applying the same criteria used for dogs, only three cats (5.5%) were adequately and continuously protected from endoparasites. All the kittens assessed during the study (n=14) were under an early deworming protocol.

Macrocyclic lactone combinations, specifically milbemycin oxime-praziquantel, were the most commonly used AH drugs in cats and kittens (72.7%) (Table 2).

3.2 Parasite control practices: Ectoparasiticides

It was found that 81.1% (n=270/312) of the interviewed owners used ectoparasitic drugs in their pets. These were given for different reasons depending on the species: in 91% of dogs an ectoparasitic drug was given as prophylaxis and the remaining 9% as a treatment for a previously diagnosed/suspected infection; in 62% of cats ectoparasitic drugs were used for prophylaxis and the remainder 38% as a treatment measure.

3.2.1 Dogs

Data showed that 92.2% (n=188) of the adult dogs were treated with ectoparasitic drugs; of these, 50.5% were treated every month (Table 3). Once again, a high percentage of respondents (42%) frequently forgot to re-administer AH drugs, thus leaving the animal unprotected for an extended period of time.

Table 3 – Ectoparasite control practices in adult dogs and cats.

	Dogs		Cats		*P* value
	n	(%)	n	(%)	
Total number of adult animals analysed	204		55		
Treated with ECTO	**188**	**(92.2)**	**29**	**(52.7)**	**<0.001**
-Treated with ECTO at least once a month	**95**	**(50.5)**	**5**	**(17.2)**	
More than once a month	7	(3.7)	-	-	
At monthly intervals throughout the year	52	(27.7)	5	(17.2)	
At monthly intervals during high risk[t] season	36	(19.1)	-	-	
-Treated with ECTO less than once a month	**93**	**(49.5)**	**24**	**(82.8)**	
At bimonthly intervals	9	(4.8)	2	(6.9)	
At 3-monthly intervals	13	(6.9)	1	(3.45)	
Once or twice during high risk[t] season	26	(13.8)	4	(13.8)	
Only Insecticide-Acaricide collar	15	(8)	1	(3.45)	
Other (irregular intervals/doesn't know)	30	(16)	16	(55.2)	
Treated with ECTO in the previous month	**82**	**(40.2)**	**9**	**(16.4)**	**0.1**
Treated continuously* ECTO	**72**	**(35.3)**	**4**	**(7.3)**	

Note: ECTO – ectoparasiticide; [t]warmest months, usually from April/May to October/November in Portugal; *In accordance with guidelines and considering the **two distinct criteria** – treated at least monthly and in the previous month. *P* values refer to statistical differences between dogs and cats.

If we consider owners' compliance to a minimum monthly regimen (either all-year round or seasonally, with spot-on formulations) and treatment in the previous month, then only 35.3% of the dogs (72/204) were adequately and continuously protected with ectoparasiticides. If we consider an all-year round continuous protection as the most secure choice for each animal, then merely 28.4% of the dogs (58/204) were uninterruptedly protected throughout the year from the main CVBD vectors (fleas, ticks, mosquitoes and sandflies), with spot-on and/or collars. On a different note, 60% of the owners who kept their dog outdoor all day did not perform adequate preventive ectoparasitic protection.

No significant statistical association was found between the frequency of treatment and owners' background or animal's information (age and breed).

Regarding puppies, 33.3% (13/39) had not been treated with ectoparasitic drugs at the time of the interview, a practice justified by the owners considering their pet's indoor restriction. Approximately half of the puppy owners (53.9%) and 80.8% of the adult dog owners were self-motivated to administer insecticide-acaricide products (P=0.008). Spot-on formulations were the most frequently used (89.4%), followed by antiparasitic collars (30.3%) and by other formulations (sprays, powders, shampoos or oral) (4.3%). Imidacloprid-permethrin spot-on combination was the most cited (59.7%) in dogs and puppies (Table 4). Considering the active substance present in collars, deltamethrin was the most common (41/57), followed by amitraz/diazinon (4/57), imidacloprid-flumethrin (1/57) and the remaining non-specified (11/57). It was noticed that 4.5% of the dogs had been vaccinated against *Leishmania infantum*,

with CaniLeish®Virbac, available in Portugal since 2011 as an additional control measure to this particular CVBD.

Table 4 - Ectoparasiticides used in adult dogs and cats.

Adult Dogs	% (95% CL)	Adult Cats	% (95% CL)
Imidacloprid+Permethrin	59.68 (52.4-66.8)	Imidacloprid	44.44 (26.9-63.4)
Fipronil/Pyriprole (comb.)	18.28 (13.2-24.4)	Fipronil (comb.)	37.04 (20.2-57.1)
Permethrin	9.14 (5.4-14.1)	Macrocyclic lactone (comb.)	14.81 (5.2-32.8)
Imidacloprid	5.91 (3-10.3)	Imidacloprid+Permethrin	3.70 (0.2-17.6)
Macrocyclic lactone (comb.).	3.23 (1.4-6.8)		
Lufenuron	1.61 (0.4-4.6)		
Indoxacarb	1.61 (0.4-4.6)		
Nitenpyram	0.54 (0-2.8)		

(ass.): includes molecule combinations; These results exclude collar formulations.

3.2.2 Cats

Only 52.7% (29/55) of the cats were treated with insecticide-acaricide products; this occurred monthly in only 17.2% (5/55) with the remainder being treated at irregular intervals (Table 3). As for kittens, 71.4% (10/14) had been treated with insecticide-acaricide products, although not at a monthly or routine basis (only sporadic application).

Imidacloprid spot-on formulation was the most common used drug (44.4%) (Table 4). There was a case (3.8%) of an owner who administered imidacloprid-permethrin spot-on formulation (only intended for dogs) on his cat. Additionally, an antiparasitic collar (drug non-specified) was only used in one cat.

3.3 Public knowledge regarding parasites and parasitic diseases

Owners were queried on their perception of possible ways their pets may contract endoparasitic diseases. The majority of the respondents mentioned food, followed by animal faeces, sand/soil/plants, arthropods and maternal-foetal transmission. Approximately one third of the respondents had no knowledge of possible infectious sources. Regarding potential sources of ectoparasitic infection in pets, most of the respondents mentioned outdoor environment, followed by direct transmission from infested animals. Merely 5.4% seem to acknowledge the role of fomite contamination (naming carpets, clothing and other fabrics) as a source of infection. We found that 85% of the respondents had never heard of the word “zoonosis.” Nonetheless, 75% recognised the concept behind it (“the transmission of parasites from pets to humans”). Also, 16% of the interviewees had already taken antiparasitic drugs themselves as a preventive measure (albendazole was the most cited drug).

Additionally, interviewees were given a list enumerating common parasitic diseases and respective causative agents and asked to identify the diseases they recognized. The three most frequently identified were "Scabies-mites/sarcoptic mange" (86.9%), followed by "Leishmaniasis-*Leishmania*" (83%) and "Toxoplasmosis-*Toxoplasma*" (64.7%).

3.4 Dog faeces removal practices

Owners were asked several questions about their habits concerning the collection of their pet's faeces in three distinct areas (path/pavement, parks, open field). Almost all owners (95.6%) stated that they collect their pet's faeces whenever it occurs on a path or pavement; 82.9% when in parks; and only 32.1% when on open land. In total, 37% of dog owners did not collect their dog's faeces in all inquired circumstances. Justifications given for not collecting faeces were: faeces considered fertilizers (43.2%); faeces located on abandoned/unreachable areas (37.7%); laziness/repulsiveness (14.5%); shame (4.6%). Reasons for dog faeces collecting were: avoid the risk of people stepping on it (57%); Public Health concerns (26%) and for social responsibility and a sense of civic duty (17%).

4. Discussion

The use of endo and ectoparasiticides as prophylaxis is an essential component in controlling the transmission of endemic, emergent or re-emergent parasites, as well as, other pathogens carried by ectoparasites. For this reason, pet owners should actively participate in this matter by giving their pets the correct prophylaxis in the prescribed intervals, as well as collecting faeces from public places, thereby, reducing environmental contamination pressure and safeguarding Public and Animal Health.

4.1 Parasite control practices: endoparasiticides

Our results regarding the use of endoparasitic drugs in dogs (89.7% dewormed at roughly 4-6 month intervals) closely mirror other international studies, namely from Finland (Pullola et al., 2006) and Switzerland (Sager et al., 2005) where 86% and 87% of dogs, respectively, were dewormed 1-2 times/year. A much higher AH administration frequency was found in Australia where 54% of dogs were dewormed quarterly (Palmer, Robertson, Traub, Rees & Thompson, 2008).

In the studies mentioned so far, veterinarians were found to be the main source of information and guidance concerning deworming practices and protocols for dogs of all ages. However, differences were observed in our study as veterinarians were considered to have a central role

regarding puppy prophylaxis, whereas their influence on adult dog treatment wasn't as significant. This fact may suggest negligence of adult dog owners to visit their practitioner in the first place or even a lack of encouragement by veterinarians, previously outlined by Traversa (2012). It would appear that both reasons ultimately result in lost opportunities for practitioners to promote regular deworming protocols. Also, Gates and Nolan (2010) showed in a retrospective study that only 13–23% of owners were questioned by veterinarians about the preventative use of drugs during routine medical appointments at the veterinary teaching hospital, which points out to a lack of discussion on this issue between veterinarians and pet owners.

Statistical associations were found when relating deworming frequency to owners' economic status. Purebred dog owners in fulltime employment were found to give AH most frequently. The fact that older dogs were dewormed less frequently is concerning especially if we take into account that their ability to fight infection may diminish with age. This is particularly concerning if we consider older dogs living with older people who may be frail, as this may increase their risk of contracting disease

Considering that only 63.6% of cats were dewormed and 36.4% were not dewormed at all, we can conclude that cat owners don't deworm their pets as often as dog owners. According to some interviewee statements, some cat owners believe that deworming their indoor cats is unnecessary. Despite the scant information available in literature about cats deworming practices, similar findings to ours were registered: 72% at 4 times/year (Mircean, Titilincu, Vasile, 2010); 63% at 2-4 times/year (Näreaho et al., 2012); 39% quarterly (Palmer et al., 2008).

Another key point of our study was to assess whether the most commonly used endoparasiticides in dogs and cats were effective, i.e., if they protect pets from the most prevalent endoparasites in the area. According to Lebre (2011), the protozoa *Cystoisospora* spp., *Giardia* spp. and *Cryptosporidium* spp. were the most prevalent endoparasites in three dog kennels surveyed in Lisbon, along with the nematodes *Ancylostoma caninum*, *Toxocara canis* and the cestode *Dipylidium caninum*. Taking into account that the AH most often used in dogs (praziquantel-pyrantel-febantel combination) is effective against such parasites, we can conclude that dogs taking this combination are protected from the most prevalent parasites circulating in the area, but not against *Cystoisospora* spp. and *Cryptosporidium* spp. Notwithstanding, in the event of a suspected parasitic infection, the practitioner should always perform a routine coprology test, not only to assess the parasitic agents and respective burden at a given point in time, but also to evaluate the efficacy of such AH drugs. The praziquantel-pyrantel-febantel combination also doesn't protect the animal against extra-intestinal parasites such as *D. immitis*, which is increasingly endemic in the central and southern regions of the

country (Cardoso et al., 2012; Alho et al., 2014). For such parasites, macrocyclic lactones are the AH drugs of choice used by 13.1% of dog owners.

With respect to cats, the most prevalent parasites found in stray cats in the metropolitan area of Lisbon were *Toxocara cati, Cystosospora felis, Ancylostoma tubaeforme, Dipylidium caninum, Uncinaria stenocephala and Toxascaris leonina* (Duarte et al., 2010). Considering that the most commonly administered AH drug in cats are macrocyclic lactones, we can infer that cats are protected against most of these parasites. However, the low frequency by which they are given (once or twice a year) does not guarantee an adequate protection of the animal against these parasitic diseases. Moreover, it should be highlighted that several of these pathogens can be transported into households by owners (via fomites, on clothing, shoe soles, etc), which increases the need for adequate parasite control and prophylaxis.

Furthermore, it is important to keep in mind that the inappropriate use of endoparasiticides may also lead to drug resistance, as documented when using pyrantel in the treatment of *Ancylostoma caninum* (Kopp et al., 2009) and in relation to macrocyclic lactones against *D. immitis* (Bowman, 2012).

4.2 Parasite control practices: ectoparasiticides

In the present study, 92.2% of the owners used ectoparasiticides on their dogs, reporting a higher result than the 56% observed in a previous nationwide survey carried out to assess *L. infantum* status in suspected dogs, in Portugal (Maia et al., 2011). In this study, approximately 30% of *Leishmania*-suspected dogs in Lisbon were protected from sandflies with the appropriate insect repellents. A similar percentage of dogs (28%) were found to be protected in our study, which is regrettable considering the endemicity of this disease in Portugal (Cardoso et al., 2012).

Additionally, only 28% of the dogs in our study were found to be protected all year-round against fleas, ticks, sandflies and mosquitoes. Although protection from Diptera is still recommended on a seasonal basis, a year-round approach is currently accepted as most prudent, considering Portugal's warm Mediterranean climate throughout the year and the high prevalence of canine leishmaniasis. The seasonal interruption of insecticide pet protection can easily lead to owners forgetting to resume treatment at the appropriate time. This is especially problematic when considering that *Phlebotomus ariasi* can survive in Portugal during 90-98% of the year. If there is an annual increase in temperature in the order of 3-6ºC *Phlebotomus perniciosus* can also become active throughout 70% of the year in the Lisbon area further aggravating this situation (Casimiro, Calheiros, Santos & Kovats, 2006).

Taking into account our results, it is important to acknowledge the conclusions made by Cardoso et al., (2012) who considered that a failure to use ectoparasiticide in dogs was a risk factor that contributed to a cumulative increased dog exposure to arthropod vectors and the pathogens they transmit, raising the levels of CVBDs.

Considering that the most used ectoparasiticide drug in dogs is a spot-on formulation of imidacloprid-permethrin, which is effective against CVBD vectors, we can conclude that most of these animals are adequately protected. Unfortunately, this formulation is only effective against *Aedes aegypti* and *Phlebotomus perniciosus* for 2-3 weeks, respectively. The imidacloprid-permethrin formulation also only provides tick repellence, unlike other ectoparasiticides that also provide tick expellence and are therefore more effective at preventing quick CVBD transmission. For this reason, a combination of different ectoparasiticide ingredients should be implemented to assure a complete protection.

4.3 Dog faeces removal practices and public knowledge about parasites and parasitic diseases

Similarly to the findings in our study, in the Netherlands 39% of the dog owners also failed to collect their pet's faeces in public places (Overgaauw et al., 2009). Taking into account the potential Public Health impact of not collecting faeces from infected animals and the potential zoonotic nature of some of the pathogens, this is regrettable but not surprising when considering the fact that a quarter of the interviewees were not aware of what a zoonosis meant and the fact that one third of the respondents were unable to cite any possible parasitic infection source. It is also possible to speculate that the percentage found in our study does not reflect owners' real behaviour, since it is a sensitive matter and the interview was conducted face to face and not anonymously. Interestingly, out of these three situations, collection from paths was far more frequent than the other situations suggesting that there may be a civic component behind faeces collection (i.e., people are more likely to collect if they perceive that their inaction may affect other people or simply the fact that they may be seen by others and judged).

If taken into account that a mere 12% of dogs were continuously and adequately dewormed in our study, we can conclude that the majority of the dogs were not only at risk of acquiring a parasitic infection, but were also a potential source of environmental contamination if they were infected. It is important to keep in mind that the most prevalent worms in dogs generally become infective 2-8 days following exposure (Ancylostomatidae) and may persist in the environment for years (Traversa, 2012). In fact, Otero et al., (2013) observed that 62.1% of all the *Toxocara* spp. eggs found in public parks and sandpits in Lisbon were embryonated and viable. This only reinforces the importance of owners' collection of their dog faeces in all public places,

irrespective of whether they personally perceive there to be a risk to Public Health. Similarly, veterinarians should be more proactive, by informing owners about ideal parasitic control schemes and more importantly ensuring they are adhered to. Evidence of this lack of action may be deduced from a study conducted by Wells (2007) who found that knowledge about the prevention of worm transmission was practically the same between animal and non-animal owners. The fact that both groups have similar levels of knowledge can possibly mean that veterinarians are not having the positive impact they could have on Animal and Public Health and consequently on One Health.

During our survey we also noticed that a large number of respondents had limited or no knowledge at all about some parasitic diseases (e.g., 88% and 100% had never heard of dirofilariosis and ancylostomosis, respectively), which could unfortunately lead to misconceptions already documented by Katagiri and Oliveira-Sequeira (2008). One example is the belief that apparently healthy pets cannot host parasites, thus leading to negligent parasite control practices, as there is no "apparent" reason to perform it. Balassiano, Campos, Menezes, and Pereira (2009) concluded that a lower level of owner education might be one factor associated with gastrointestinal parasite infection in dogs.

5. Conclusions

Despite the large number of potent and selective antiparasitic drugs available nowadays, parasitic diseases remain frequent in companion animals. Although the majority of pet owners give antiparasitic drugs, our results show that this occurs at irregular intervals, which renders them ineffective. The general lack of owner's knowledge observed regarding zoonotic diseases and sources of parasitic infection, demonstrates a failed opportunity on the part of veterinarians, as the main source of owner information, to raise public awareness.

We believe that to achieve a successful and sustained management of parasitic infections, a more integrated approach should be taken particularly during routine consultations where emphasis should be made on the importance of performing adequate prophylaxis throughout the pet's life, regardless of its age.

Apart from endoparasiticides one must also not neglect the relevance of ectoparasiticides, which now, more than ever, play a crucial role in the overall parasite control picture. Ectoparasites are no longer a modest undesired and uncomfortable problem within the household. Current climatic, political and demographic changes make them a growing concern in the transmission of pathogens, many of which may constitute a zoonotic hazard. Seasonal administration of insecticides-acaricides may not be sufficient in a constant ever-changing

world. In other words, the implementation of continued ectoparasiticide prophylaxis is of the utmost importance, especially in high-risk CVBD areas, namely Southern European countries. We hope that the obtained data might encourage veterinarians to engage owners more frequently on this subject, thereby enhancing public knowledge and overcoming owners rooted misconceptions. This may ultimately help to promote higher Public Health standards and reinforce the need of effective preventive antiparasitic control measures in companion animals.

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CHAPTER 8

The current situation of two major canid heartworms in Portugal

Chapter 8, Scientific publication 1

Dirofilaria immitis and *Angiostrongylus vasorum*: the current situation of two major canid heartworms in Portugal

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Abstract

Cardiopulmonary nematodes are life-threatening pet parasites increasingly reported throughout Europe, with overlapping endemic areas. *Dirofilaria immitis* is a mosquito-borne whilst *Angiostrongylus vasorum* is a snail-borne pathogen. Both adult nematodes reside in the pulmonary arteries and right cardiac ventricle of domestic and wild canids, causing a wide spectrum of clinical features that may lead to death. Information about the prevalence and distribution of cardiopulmonary parasites is essential for the control of animal diseases and, in the case of *D. immitis,* for the control of potentially associated illnesses in humans. However, in Portugal, heartworm studies are limited to few surveys and case reports, possibly underestimating the relevance of these nematodes. The present work reviews the data on cardiopulmonary dirofilariosis and angiostrongylosis in dogs in Portugal, providing a comprehensive update of the epidemiological situation during the past 20 years.

Keywords: *Angiostrongylus vasorum,* cardiopulmonary nematodes, *Dirofilaria immitis*, dog, epidemiology, Portugal.

1. Introduction

Cardiopulmonary nematodes are severe and life-threatening parasites of pets, which have increasingly been reported throughout Europe, with overlapping areas of endemicity, as reviewed by Traversa, Di Cesare and Conboy (2010). Several factors may account for the expansion, including faster and incremented global transports (with the concurrent movement or relocation of infected animals from endemic to non-endemic areas), demographic and political changes, urbanization, increasing density of vulpine reservoir host populations, climate changes, drug resistance among vectors and pathogens, and the availability of better diagnostic tools, as reviewed by Colwell, Dantas-Torres and Otranto (2011) and Otranto et al., (2013).

Dirofilaria immitis (Leidy, 1856) (Filarioidea, Onchocercidae) is the causative agent of canine cardiopulmonary dirofilariosis (CCPD), also known as heartworm disease. It is a potentially zoonotic vector-borne pathogen, transmitted by multiple species of mosquitoes (Culicidae). Domestic and wild canids constitute the natural host of this nematode, although infection may also occur in other mammalian species, i.e. cats, ferrets, lions, otters, as reviewed by McCall, Genchi, Kramer, Guerrero and Venco (2008) and Simón et al., (2012). In humans, *D. immitis* is an aberrant parasite that may cause pulmonary nodules, occasionally misdiagnosed as malignant lesions (Muro & Cordero, 2001). *Angiostrongylus vasorum* (Baillet, 1866) (Metastrongyloidea, Angiostrongylidae), also known as the French heartworm, is the causative agent of canine angiostrongylosis (CA). This nematode is transmitted by gastropod molluscs (Guilhon & Cens, 1973) and is not considered to be zoonotic. Adult stages of both nematodes reside in the pulmonary arteries and right cardiac ventricle of canids, causing a wide spectrum of clinical problems, ranging from mild to severe forms, which can inclusively be fatal. Clinical signs such as cough and dyspnoea, as well as non-specific manifestations like depression, weight loss, vomiting and anorexia, are the most common ones. In the particular case of *A. vasorum* infection, haemorrhagic diathesis and disease of the central nervous system may also occur (Chapman, Boag, Guitian & Boswood, 2004; reviewed by Koch & Willesen, 2009; and Simón et al., 2012).

Regarding their geographical distribution, *D. immitis* is present in tropical, subtropical and temperate regions throughout the world, including canine populations from Europe, Asia, Australia, Africa and America (Simón, Morchón, González-Miguel, Marcos-Atxutegi & Siles-Lucas 2009). In the European continent, the highest prevalence of CCPD has been reported in the southern countries (historically considered to be endemic or hyperendemic), although studies suggest a recent expansion towards central and northern Europe, as reviewed by Morchón, Carretón, González-Miguel and Mellado-Hernández (2012) and Simón et al., (2012).

On the other hand, *A. vasorum* has been characterized by a patchy geographic distribution, with isolated foci, surrounded by areas with only sporadic occurrences (Morgan, Jefferies, Krajewski, Ward & Shaw, 2009). Nevertheless, *A. vasorum* range is expanding and is now recognized as having a wide distribution, occurring mostly in Europe, but also in Africa, Atlantic Canada and South America, as reviewed by Bolt, Monrad, Koch and Jensen (1994) and Koch and Willesen (2009).

Information about the prevalence and distribution of cardiopulmonary parasites is essential for the control of animal and potentially associated human diseases, particularly considering their growing incidence and clinical severity. However, in Portugal, accurate data on both illnesses is scarce and limited to a few studies and case reports. The present work aimed at reviewing the data of cardiopulmonary dirofilariosis and angiostrongylosis in dogs in Portugal in the past 20 years, in order to provide a comprehensive update of the current epidemiological situation.

2. Prevalence of *D. immitis* in dogs in Portugal

Portugal is historically regarded as a country where canine dirofilariosis by *D. immitis* is endemic. It is one of the warmest countries in Europe, with mainland annual average temperatures varying from 8–12°C, a favourable climate for vector development, breeding and survival.

The first study carried out in the country to assess *Dirofilaria* spp. occurrence showed that CCPD was prevalent in several regions of southern Portugal, as well as on Madeira island, where the highest prevalence was registered, as reviewed by Araújo (1996). However, this study was based exclusively on microfilariae detection, underestimating occult infections, and without differentiating other canine filarial species. It's important to highlight that other canine filarial occur in Portugal, i.e. *Acanthocheilonema dracunculoides, Acanthocheilonema reconditum* and *Dirofilaria repens,* thus microfilariae need to be distinguished from those of *D. immitis* (Menn, Lorentz & Naucke, 2010; Magnis et al., 2013; Maia, Lorentz, Cardoso, Otranto & Naucke, 2016).

Later on, in a national serological survey based on the detection of *D. immitis* antigen, the prevalence in Portugal ranged from 3.6% in apparently healthy dogs to 8.9% in clinically suspect dogs (Cardoso, Mendão & Madeira de Carvalho, 2012). However, some areas registered a higher prevalence, namely: Faro district coterminous with the Algarve region (9.4%) (Maia et al., 2015); Santarém district (13.2%) (Alho et al., 2014b); Setúbal district (24.8%) (Alho et al., 2014b); Coimbra district and Figueira da Foz municipality, where prevalence reached 25-26% (Alho et al., 2014b; Vieira et al., 2014, 2015). This high prevalence might be explained by the fact that these regions are estuarine areas, located near Guadiana,

Sado, Tejo and Mondego rivers with abundant wetlands and rice fields, that represent favourable breeding sites for mosquito populations. Additionally, with as much as 40% of infected dogs (Cardoso et al., 2012), Madeira island is a hyperendemic area for CCPD, potentially attributed to its subtropical climate (Table 1).

Table 1 – Review on the occurrence of *Dirofilaria immitis* infection in dogs in Portugal.

Study year(s)	Number of dogs	Detection method	Regions/districts	Prevalence (%)	References
1989-1990	NA	Knott's test	Alentejo (R) Algarve (R) Madeira (R) Ribatejo (R)	16.5 12.0 30.0 16.7	Araújo (1996)
2005-2010	271	*D. immitis* antigen kit + fresh blood drop	Aveiro (D)	9.3	Rendall-Rocha, et al. (2012)
2009-2011	304	*D. immitis* antigen kit + Knott's test + acid phosphatase staining	Figueira da Foz (municipality)	27.3	Vieira et al. (2014)
2010-2011	1185	*D. immitis* antigen kit	Alentejo (R) Algarve (R) Azores (R) Centre (R) Lisbon (R) Madeira (R) North (R)	4.7 / 4.0* 5.1 / 17.1* 0.0 / 0.0* 0.9 / 7.4* 2.4 / 5.8* 40 / NA* 2.9 / 3.4*	Cardoso et al. (2012)
2010-2011	386	*D. immitis* antigen kit	Aveiro (D) Braga (D) Bragança (D) Coimbra (D) Oporto (D) Viana do Castelo (D) Vila Real (D) Viseu (D)	6.8 0.0 0.0 8.8 0.0 2.1 0.0 0.0	Vieira et al. (2015)
2011-2013	696	*D. immitis* antigen kit + Knott's test + acid phosphatase staining	Coimbra (D) Santarém (D) Setúbal (D)	13.8 13.2 24.8	Alho et al. (2014b)
2011-2014	170	*D. immitis* antigen kit	Algarve (R)	9.4	Maia et al. (2015)
2014	248	*D. immitis* antigen kit + Knott's test	Beja (D) Bragança (D) Castelo Branco (D) Évora (D) Faro (D) Guarda (D) Portalegre (D)	8.9 0.0 2.5 0.0 2.7 6.7 0.0	Alho et al. (2014a)
2014-2015	100	*D. immitis* antigen kit	Aveiro (D) Azores (R) Beja (D) Leiria (D) Lisbon (D) Madeira (D) Setúbal (D)	0.0 0.0 0.0 0.0 0.0 0.0 0.0	Alho et al. (2016b)

Note: *The first prevalence value was obtained in apparently healthy dogs and the second in clinically suspect dogs; NA – not available/assessed; D – district; R – region. The *D. immitis* antigen kit used were: WITNESS® Dirofilaria or Fastest® HW Antigen in Rendall-Rocha, et al. (2012); SNAP®4Dx® in Cardoso et al. (2012); IDEXX SNAP® in Vieira et al. (2014); PetChek® canine heartworm antigen test in Maia et al. (2015); and Uranotest Dirofilaria® in Vieira et al. (2015); WITNESS® Dirofilaria in Alho et al. (2014a; 2014b, 2016b).

Although prevalence varies geographically, evidence of *D. immitis* infection was found in dogs from almost all regions of Portugal. According to Cardoso et al., (2012), a southerly trend of positivity to canine vector-borne agents is found in the country, with southern regions such as the Algarve, Alentejo, Lisbon and Madeira registering higher prevalence than the North. This is possibly due to bioclimatic and ecological factors, as temperatures tend to be higher in southern areas, thus providing more favourable conditions for vector proliferation.

Overall, the prevalence in Portugal is similar to those in other southern European countries with Mediterranean temperate climates, such as Spain, Italy, France, Greece and Turkey (Genchi, Rinaldi, Cascone, Mortarino & Cringoli, 2005; reviewed by Morchón et al., 2012). In Spain, for instance, *D. immitis* has a wide distribution, but its prevalence is higher in irrigated areas, as well as in southern locations, such as Cadiz (12%), Córdoba (18%), Badajoz (8–14%) and Alicante (13%), with the maximum levels reported in the province of Huelva (36.7%), as reviewed by Guerrero, Rojo-Vazquéz and Rodenas (1989); Ortega-Mora, Gómez-Bautista, Rojo-Vazquéz, Rodenas and Guerrero 1991). Comparably to Madeira, the Canary Islands of Tenerife (20.0%) and Gran Canária (36.0%) are also endemic or hyperendemic for CCPD (Guerrero et al., 1992).

Nevertheless, epidemiological studies are suggesting a change to the distribution pattern of the parasite occurrence, with a trend of spreading to northern and Eastern Europe (Morchón et al., 2012).

3. Prevalence of *A. vasorum* in dogs in Portugal

In the last few years, some case reports described *A. vasorum* infection in Portuguese domestic dogs (Pötz, 2006; as reviewed by Madeira de Carvalho, Pereira da Fonseca, Gomes & Meireles, 2009; Madeira de Carvalho et al., 2013). A study using the Baermann technique on faecal samples found a prevalence of 2.0% in the Lisbon area (Nabais et al., 2014).

A mass screen survey conducted in dogs all over mainland Portugal showed a total of 0.66% (6/906) positives for both *A. vasorum* circulating antigen and specific antibodies in serum by enzyme-linked immunosorbent assays (ELISAs). These animals were detected in northern, central and southern areas of Portugal, indicating active infections widely distributed throughout the country. Additionally, 1.32% (12/906) were *A. vasorum* antibody-positive, suggesting previous exposure to the parasite (Alho et al., 2016c). In a study conducted in hundred Portuguese military dogs tested by the same ELISAs, 5% were seropositive for *A. vasorum* antibodies, with positives in Aveiro, Lisbon and Setúbal (Alho et al., 2016b). In other studies performed using a commercial antigen kit (AngioDetect^{TM}), no positive dogs were found in the assessed geographic areas (Maia et al., 2015; Serrão et al., 2016) (Table 2).

Table 2 – Review on the occurrence of *Angiostrongylus vasorum* infection in dogs in Portugal.

Study year(s)	Number of dogs	Detection method	Regions/districts	Prevalence (%)	References
2011	50	Baermann technique	Lisbon(D)	2.0	Nabais et al. (2014)
2011-2014	906	ELISAs for *A. vasorum* circulating antigens and parasite-specific antibodies	Beja (D) Braga (D) Bragança (D) Castelo Branco (D) Coimbra (D) Évora (D) Faro (D) Guarda (D) Lisbon (D) Portalegre (D) Oporto (D) Santarém (D) Setúbal (D) Viana do Castelo (D) Vila Real (D) Viseu (D)	0.0 2.2* 3.8* 0.0 3.4* 1.1+ 0.0 2.7+ 0.0 0.0 0.0 0.0 2.2*1.6 + 2.9* 0.0 2.0* 2.0+	Alho et al., (2016c)
2011-2014	120	*A. vasorum* antigen kit (Angio Detect™)	Algarve (R)	0.0	Maia et al. (2015)
2012-2014	400	*A. vasorum* antigen kit (Angio Detect™)	Alentejo (R) and Algarve (R) Centre (R) Lisbon (R) North (R)	0.0 0.0 0.0 0.0	Serrão et al. (2016)
2014-2015	100	ELISAs for *A. vasorum* circulating antigens and parasite-specific antibodies	Aveiro (D) Azores (R) Beja (D) Leiria (D) Lisbon (D) Madeira (R) Setúbal (D)	11.1* 0.0 0.0 0.0 6.7* 0.0 4.8* 4.8+	Alho et al. (2016b)

*Antibody-positive only; +Simultaneously positive for circulating *A. vasorum* antigens and antibodies against the parasite; D – district; R – region; The *A. vasorum* antigen kit used was AngioDetect™ in Maia et al. (2015) and Serrão et al. (2016).

Different studies may be not comparable, as they were performed with distinct dog populations (owned vs. stray, healthy vs. clinically suspect) and/or with different diagnostic tests (e.g. detection of antibodies, antigens in blood or first stage larvae in faeces) with distinct performances. On the other hand, differences may be due to the geographical areas assessed, as some locations might be more suitable for *A. vasorum* establishment. Furthermore, dogs' age (younger dogs are at higher risk due to their immature immune system and/or their greater leaning to eat gastropods) might constitute individual risk factors (Chapman et al., 2004; as reviewed by Elsheikha, Holmes, Wright, Morgan & Lacher, 2014).

Serological methods have been proven to be a valid diagnostic alternative for both individual and massive screening of dog populations, allowing the rapid detection of infection, shortly before or contemporaneously with patency (Schnyder, Tanner, Webster, Barutzki & Deplazes,

2011; Schucan et al. 2012; Schnyder, Jefferies, Schucan, Morgan & Deplazes, 2015a). Overall, the seroprevalence of CA in Portugal (0.66%; regarded as the positivity for both *A. vasorum* circulating antigen and specific antibodies against the parasite), compared to other European countries, is slightly higher than the ones found in Germany (0.3%) (Schnyder, Schaper, Bilbrough, Morgan & Deplazes, 2013a) or Poland (0.51%) (Schnyder et al., 2013b), and lower than in Hungary (1.36%) (Schnyder, Schaper, Lukács, Hornok & Farkas, 2015b), United Kingdom (0.97%) (Schnyder et al., 2013a), Switzerland (0.96%) (Lurati, Deplazes, Hegglin & Schnyder, 2015) and Italy (0.8-0.9%) (Guardone, Schnyder, Macchioni, Deplazes & Magi, 2013), but differences are not statistically significant.

4. Infection in vectors and in intermediate hosts in Portugal

The involvement of vector mosquitoes in the life cycle of *Dirofilaria* spp. makes their transmission and distribution susceptible to climate changes, as well as to rapid and significant variations in defined geographic regions. According to Ferreira et al., (2015), *Culex theileri*, *Culex pipiens*, *Anopheles maculipennis* s.l., *Anopheles atroparvus*, *Aedes caspius* and *Aedes detritus* s.l. were found to be naturally infected with *D. immitis* in mainland Portugal. This is worrying if we consider that out of the 41 species of mosquitoes identified in continental Portugal (Ribeiro, Ramos, Pires & Capela, 1988), *An. maculipennis* s.l., *Cx. pipiens* s.l., *Cx. theileri* and *Ae. caspius* were the most abundant and broadly distributed mosquitoes (Almeida et al., 2008) and have been proven to be vectors of *D. immitis*.

Regarding *A. vasorum*, a diversified spectrum of gastropods has been described as intermediate hosts. In Portugal, the *Arion rufus* and *Deroceras laeve* slugs are known to be *A. vasorum* suitable intermediate hosts, and have inclusively been described in distinct parts of the national territory (Grewal, Grewal, Tan & Adams, 2003; Bank, 2011). Also, frogs (*Rana temporaria*) may potentially play a role as paratenic hosts (Bolt, Monrad, Frandsen, Henriksen & Dietz, 1993).

5. Transmission risk of *D. immitis* and *A. vasorum* in Portugal

The transmission of *D. immitis* depends on several factors, such as: sufficient numbers of infected and microfilaremic dogs, competent mosquito species and suitable climatic conditions that allow the extrinsic incubation of the parasites in the vector mosquitoes (Medlock, Barrass, Kerrod, Taylor & Leach, 2007). Growing degree day-based forecast models have been used to predict the occurrence and seasonality of *D. immitis* in different parts of the world (Genchi et al., 2005; Genchi, Rinaldi, Mortarino, Genchi & Cringoli, 2009). In a preliminary analysis conducted over a decade at five meteorological stations in Portugal, Madeira Island was found

to be the area registering the highest number of days with suitable conditions for *D. immitis* transmission (an average of 209.9 days per year). Faro was the second one (175.2 days/year), followed by Lisbon (163.5 days/year), Azores (140 days/year) and Oporto (117.2 days/year). The average risk period was 8 months/year in Madeira, 6.9 in Faro, 6.4 in Lisbon, 5.6 in Azores and 5 months/year in Oporto (Alho et al., 2014c). Overall, this study evidenced that in Portugal the risk of *D. immitis* transmission starts earlier and lasts far beyond the warmest months of the year. The periods obtained were lengthier than those found previously by Genchi et al., (2005). This is in line with the "seasonality paradigm" of vector-borne diseases, which shows that their occurrence is no longer a seasonal phenomenon (Otranto et al., 2013). Thus, effective protection against vectors over an extended period is needed. A continued year-round prophylactic approach is recommended, considering the high prevalence of *D. immitis* and Portugal's warm climate throughout the year. Furthermore, as a consequence of climate changes, we are facing an increasing transmission risk of vector-borne pathogens in the country (Casimiro, Calheiros, Santos & Kovats, 2006), which may become worse with the spread of *Aedes albopictus* (the Asian tiger mosquito that is adapted to colder climates) and the introduction of *Aedes koreicus* which is enhancing the spread of *D. immitis* in endemic and non-endemic areas (Montarsi et al., 2015).

Climate is hypothesised to influence the transmission of *A. vasorum*, as the activity of its intermediate host species and its population dynamic are highly dependent on temperature and moisture conditions (Morgan et al., 2009). However, due to a paucity of experimental data on the effect of climate on *A. vasorum* development and transmission, few studies have been conducted to predict the extent to which current and future distributions might be limited by climate. A simulation based on the observed distribution of *A. vasorum* in Europe and eco-climatic similarities predicted highly suitable areas in the northern regions of Portugal (characterized by average low temperatures and high humidity) and no or low suitability in the central and southern parts of the country, where temperatures are frequently higher and humidity lower (Morgan et al., 2009).

Nevertheless, if we consider the recent erratic spread of CA from classic hotspots to previously uninfected areas and the remarkable spread of CCPD in recent times throughout Europe, it is evident that it is rather challenging to determine whether an animal is at a high or low risk of infection solely based on its location. Moreover, the results obtained so far regarding CA show that infected dogs can be found either in northern, central and southern regions (Alho et al., 2016c), contradicting the above mentioned epidemiological model concerning the occurrence of this lungworm in Portugal (Morgan et al., 2009).

6. Prevalence of *D. immitis* and *A. vasorum* in wild animals in Portugal

Many wild animals act as sylvatic reservoirs for *Dirofilaria* spp. by sustaining the transmission of these parasites to companion animals. In Portugal, the prevalence of *D. immitis* detected by necropsy of red foxes (*Vulpes vulpes*) ranges from 3.2% in northern-centre locations, such as Coimbra district (Eira, Vingada, Torres & Miquel, 2006), to 11.8% in southern and central districts, such as Santarém and Setúbal (Carvalho-Varela & Marcos, 1993). Additionally, in a national serological survey conducted in red foxes in Portugal, 8.5% (10/118) were positive for *D. immitis* circulating antigens, with positive animals found in northern and southern areas (Alho et al., 2016a). *D. immitis* has also been reported in three Eurasian otters (*Lutra lutra*) in Portuguese natural freshwater habitats (Torres et al., 2004; Saraiva et al., 2012), and more recently in a collection of pinnipeds: common seals (*Phoca vitulina*), California sea lions (*Zalophus californianus*) and South African fur seals (*Arctocephalus pusillus pusillus*), housed in an oceanographic park in the Algarve, southern region (Alho et al., 2017).

In Portugal, *A. vasorum* was first identified during the necropsy of one out of 306 red foxes (*V. vulpes silacea*) sampled between 1970-1987, mostly from the coastal central and southern regions of the country (Carvalho-Varela & Marcos, 1993). More recently, an *A. vasorum* infection prevalence of 16.1% (10/62) was found by necropsy of red foxes collected by hunters from central Portugal (Eira et al., 2006). In addition, *A. vasorum* was also detected in 7.1% (2/28) of red fox faecal samples collected in western-central zone (Figueiredo, Oliveira, Madeira de Carvalho, Fonseca & Torres, 2016).

7. Prevention practices and control measures in dogs in Portugal

Despite the availability of a variety of antiparasitic prophylactic measures, studies show that although the majority of Portuguese pet owners give antiparasitic drugs to their dogs, most of them deworm at irregular and consequently ineffective intervals. On a pet owners' questionnaire conducted at a veterinary hospital in Portugal, only 11.8% of the dogs were under the recommended endoparasitic treatment (i.e. quarterly, at least) and only 28.4% were continuously protected throughout the year from vector-borne agents. Additionally, 60% of the owners who kept their dogs outdoors the whole day, did not perform adequate ectoparasitic prevention on their animals (Matos, Alho, Owen, Nunes & Madeira de Carvalho, 2015). It is important to highlight that for *D. immitis* control in dogs, the prophylactic use of macrocyclic lactone-based products is vital. Monthly administration of topical or oral macrocyclic lactones (or of 6 months slow-release formulation) has shown to be effective for prevention and is recommended. Control measures to prevent mosquito bites are also crucial to reduce *D. immitis* infection, namely: regular application of mosquito repellents, emptying standing water

collections, installation of window screens and avoidance of areas and periods of the day when mosquitoes are most active (ESCCAP, 2017), although measures cannot guarantee a hundred percent protection. To control *A. vasorum*, strategies indicated to reduce the risk of infection with third-stage larvae include: preventing dogs from ingesting snails or slugs, avoid leaving dogs outside the house or feeding there, and regular cleaning of dogs' bowls and toys to make them less accessible to slugs/snails (Elsheikha et al., 2014).

Furthermore, the preventive safety measures partaken by dog owners in public places are still insufficient, with 37% answering they did not collect their dogs' faces in all public places (Matos et al., 2015). This may potentially contribute to environmental contamination with, among others, first stage larvae of *A. vasorum*. The need to implement environmental control measures (namely dog faeces removal from public places) along with effective worm control in dogs is fundamental, to minimize environmental contamination and safeguard public and animal health (ESCCAP, 2017).

The level of public knowledge about parasites and parasitic diseases is still low in the country, i.e. 88% had never heard of "dirofilariosis/heartworm disease" and 85% had never heard of "zoonosis". One third of the owners were unable to cite any possible source of parasitic infection (Matos et al., 2015). In another survey conducted in Portugal, although 56.5% of the owners had heard of "zoonosis/zoonoses", only 35.2% knew their real meaning and 50.8% were unaware of "dirofilariosis/heartworm disease" (Pereira et al., 2016). The general lack of owners' knowledge regarding parasitic infections and adequate control schemes highlights the key role of veterinarians in providing owner information and raising public awareness. The implementation of continued ectoparasiticide prophylaxis is crucial, especially in high-risk canine vector-borne disease areas, like southern European countries, including Portugal. It is also relevant to perform screenings for *D. immitis* and *A. vasorum* infections on a regular basis, especially given the chronic progression and subclinical character of the induced diseases (McCall et al., 2008; Koch & Willesen, 2009). For *D. immitis*, dogs should be checked for both circulating antigens and blood microfilariae (Knott's test) at the beginning of each preventative annual treatment (ESCCAP, 2017). For *A. vasorum*, dogs should be checked for the presence of first stage larvae in faeces collected over three consecutive days, using the Baermann migration technique (ESCCAP, 2017), or, in clinically suspect animals, the commercial serological tests for the detection of the circulating *A. vasorum* antigens (Schnyder, Stebler, Naucke, Lorentz & Deplazes, 2014).

According to a questionnaire conducted to examine veterinary awareness and perceptions on CCPD in Western Europe (including France, Germany, Italy, Netherlands, Spain, United Kingdom), 10% of the practitioners from non-endemic areas and 12% of those from endemic

areas reported an increasing number of cases over the previous five years (Genchi, Bowman & Drake, 2014). Most practitioners were not aware of the European Scientific Counsel Companion Animal Parasites (ESCCAP) guidelines concerning the prevention of heartworm in Europe, with 54% (317/584) not aware, 37% (213/584) slightly/moderately aware; and only 9% (54/584) quite/very aware of the guidelines (Genchi et al., 2014). Commitment is needed to increase the awareness concerning ESCCAP aims and respective guidelines to rise its implementation in clinical practice.

No surveys on CA have been performed up to now in Portugal to assess owner's or practitioner's knowledge regarding this disease.

8. Prevalence of *D. immitis* in humans in Portugal

Previously considered a rare disease, dirofilariosis in humans has been increasingly reported, as reviewed by Pampiglione, Canestri Trotti and Rivasi (1995). Although *D. repens* is the most important cause of human filarial infections in Europe (even in areas where *D. immitis* predominates), cases of human infection by *D. immitis* have also been reported. Pulmonary dirofilariosis by *D. immitis* is mostly characterized by the presence of single (or, more rarely, multiple) nodules located in peripheral areas, such as the subpleural region of human patients (Muro & Cordero, 2001). Infection is usually asymptomatic, although cough, thoracic pain, haemoptysis, dyspnoea, fever and malaise have been reported (Muro & Cordero, 2001).

Knowledge, attitudes and practices of medical clinicians regarding human dirofilariosis were assessed by an online questionnaire. Some findings were of major relevance, namely the 38.1% of 84 who had never heard of dirofilariosis. Of those who did, approximately half of them evaluated their knowledge as insufficient or non-existent. Only 45.2% of the respondents associated dirofilariosis with a vector-borne disease. Therefore, due to lack of awareness amongst health professionals and difficulties inherent to parasite identification, human dirofilariosis is probably underdiagnosed. Despite the endemicity of *D. immitis* in dogs, only two cases of pulmonary nodules by *D. immitis* have been reported so far in Portugal, as reviewed by Araújo et al. (1996). According to the clinicians' questionnaire, 10 additional cases of *Dirofilaria* spp. infection (seven cutaneous, two pulmonary and one ophthalmic) were diagnosed in humans by serology or histology in Portugal over the previous five years, although not published. These data show that *Dirofilaria* spp. infection is still a relatively unknown disease for clinicians, highlighting the need for more information also in the medical community (Belo, Afonso & Gonçalves, 2014).

9. Discussion

Despite current research and advances on CCPD and CA, there is still insufficient data and limited understanding on the spread of both diseases to predict further range expansions. Some difficulties and misconceptions regarding *D. immitis* and *A. vasorum* may impair their appropriate diagnosis. Both diseases may present a wide spectrum of clinical manifestations that can lead clinicians to overlook infections with these two pathogens, inducing to treatments for other conditions that are generally considered to be more prevalent. Additionally, some clinicians tend to perform therapeutic treatments without attempting a definitive diagnosis, disregarding important evidence on local epidemiological risk and consequent data on treatment and prevention regimens (Elsheikha et al., 2014). Diagnostic techniques, such as Knott's test or Baermann examination, should be more routinely used in endemic areas, to increase the active surveillance of both infections.

Although exposure may differ according to the geographical region of Portugal, the likelihood of both infections is considerable nationwide. Taking into account the ongoing changes in climate and ecosystems, dog exposure to infection seems likely to increase in the future, in either endemic and non-endemic areas. Therefore, it is crucial to alert veterinarians to take canine cardiopulmonary nematodes into consideration in their diagnostic routine. Bearing in mind the impact that heartworms may have on animal health, the zoonotic potential of *D. immitis* and the geographical range trend of both infections, it is vital to adopt effective prophylactic and adequate vector control measures, in order to prevent the spread of both diseases.

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CHAPTER 9

General Discussion and Conclusions

9.1 General discussion

In 2012, when this PhD project was designed, accurate data on the prevalence, distribution and epidemiological pattern of canine dirofilariosis and angiostrongylosis in Portugal was scarce, outdated and limited to few studies and case reports. Additionally, the little information available was published in Portuguese journals or in proceedings from national scientific meetings and was therefore, not readily available to the international scientific community. This lack of knowledge was hampering disease awareness and the implementation of effective prevention and control strategies by veterinarians, health authorities and pet owners.

Canine cardiopulmonary dirofilariosis was known to be prevalent in some regions of northern and southern Portugal, as well as on Madeira Island (Pereira da Fonseca et al., 1991; reviewed by Araújo, 1996; Clemente, 1996; Santos et al., 2000). However, studies were exclusively based on microfilariae detection, underestimating occult infections. Furthermore, there was no data relating to other *Dirofilaria* species circulating in the country, like *D. repens*. Regarding canine angiostrongylosis, its occurrence was limited to a few case reports, diagnosed through the visualization of *A. vasorum* L1 in faecal and respiratory samples using Baermann's test or by the identification of adults upon post-mortem examination (Pötz, 2006; Madeira de Carvalho et al., 2009). However, morphological identification of larvae using the Baermann's test has important limitations (absence of larvae during the pre-patent period, intermittent larval excretion, limited sensitivity on a single faecal examination, occurrence of mixed lungworm infections and its laboriousness), particularly when used in large-scale epidemiological studies. The ground-breaking development of new diagnostic techniques, such as the serological and molecular testing, proved to be more reliable and sensitive, improving detection rates and reducing the time needed to diagnose dirofilariosis and angiostrongylosis in canids. These methods became an alternative for both individual and massive screening of canid populations, allowing the rapid detection of infection, shortly before or contemporaneously with patency (Grieve, 1987; Brunner, Hendrix, Blagburn & Hanrahan, 1988; Rishniw et al., 2006; Schnyder et al., 2011b; Schucan et al., 2012; Schnyder et al., 2014). For all the above-mentioned reasons and using the new sensitive and specific diagnosing techniques, in Chapter 2, the author aimed to characterize and update the current situation of *D. immitis* and *A. vasorum* in domestic canids in Portugal.

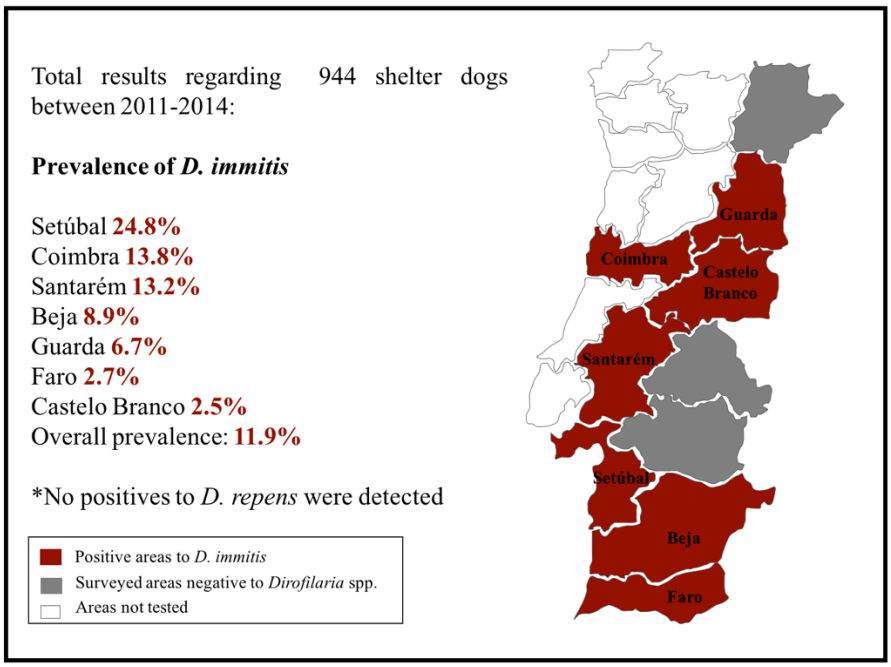
In Chapter 2, scientific publication 1, the prevalence of *Dirofilaria* spp. infection was assessed in shelter dogs from three endemic coastal regions of central Portugal (Coimbra, Santarém and Setúbal), over three consecutive years (2011, 2012 and 2013). Overall, Setúbal registered the highest prevalence (24.8%), followed by Coimbra (13.8%) and Santarém (13.2%), with a global

prevalence of 15.1%. *D. immitis* was detected in all three districts, from 2011 to 2013, with an increased prevalence from 2011 (15.3%) to 2013 (17.1%). These results confirmed the endemicity of *D. immitis* and suggested an increased transmission of canine cardiopulmonary dirofilariosis in Portugal.

In Chapter 2, scientific publication 2, given the scarcity of data on *Dirofilaria* spp. in the cross-border and inland regions of Portugal and Spain, the study was extended. In a survey performed in 2014, seven additional districts were studied (Beja, Bragança, Castelo Branco, Évora, Faro, Guarda and Portalegre), involving further 248 canids. Results showed that Beja was the district with the highest prevalence of *D. immitis* (8.9%), followed by Guarda (6.7%), Faro (2.7%) and Castelo Branco (2.5%). No positive cases of *D. immitis* infection were recorded in Bragança, Évora and Portalegre.

Overall, these two studies involved 944 shelter dogs, from 10 districts, surveyed between 2011 and 2014. A total of 11.9% of dogs were positive to *D. immitis* (Fig. 9).

Figure 9 - Overall prevalence of canine dirofilariosis in dogs from Portugal. (original).



The prevalence registered was higher than expected when compared with previous studies, such as the national serological survey of CVBDs conducted by Cardoso, Mendão and Madeira de Carvalho (2012), who reported a 3.6% prevalence of *D. immitis* infection in apparently healthy owned dogs and a 8.9% prevalence of *D. immitis* in clinically suspect owned dogs. The higher prevalence detected in our study might be partially explained by the fact that it was performed with shelter dogs, which usually do not receive prophylactic heartworm treatments and are

therefore, at a higher risk of infection. Another factor is the recent socioeconomic crisis in Portugal that resulted in a reduction in prophylactic treatments, particularly those directed against vectors, which may have contributed to this increased prevalence (Madeira de Carvalho et al., 2013).

An overall prevalence of 11.9% was found for *D. immitis* infection in dogs from Portugal, showing an increment in comparison to previous studies.

These results are consistent with the data obtained by Ferreira et al., (2015), who assessed *Dirofilaria* spp. in mosquitoes in the same areas and periods, and only detected *D. immitis.* Other canine filarial species found during the present survey were *A. dracunculoides* and *A. reconditum. Dirofilaria repens* was not found by any of the diagnostic methods. Although *D. repens* was not identified in our study, a presumable autochthonous *D. repens* infection was reported in 2016, in a dog from the Algarve region (Maia, Lorentz, Cardoso, Otranto & Naucke, 2016a). Its presence was expected considering the previous reports of *D. repens* in other neighbouring Mediterranean countries, including Spain (Rojo-Vázquez, Valcárcel, Guerrero & Gómez, 1990), France (Chauve, 1997) and Italy (Tarello, 2010), some of them registering high prevalences. Although only one case of *D. repens* has been found in Portugal, it is a matter of concern, as this species has been implicated in an increasing number of reports of human dirofilariasis in Europe (Genchi et al., 2011).

Overall, we can say that presently, both *D. immitis* and *D. repens* exist in Portugal, although *D. immitis* (the most virulent canid filarial species) is more prevalent and remains, so far, the main etiological agent of dirofilariosis in the country. Although prevalence varies geographically, we now know that canine CPD is prevalent in several regions of Portugal and that regional prevalences vary depending on respective climatic conditions and vector densities. A southerly trend of positivity for canine vector-borne agents appears evident in the country, with southern regions such as the Algarve, registering higher prevalence than the North. This is possibly due to bioclimatic and ecological factors, such as higher temperatures in southern areas, that may provide more favourable conditions for vector proliferation. Overall, the prevalence in Portugal is similar to those in other southern European countries with Mediterranean temperate climates, such as Spain, Italy, France, Greece and Turkey (Genchi et al., 2005; reviewed by Morchón et al., (2012).

Currently, both *D. immitis* and *D. repens* are present in Portugal, although *D. immitis* is more prevalent and remains, so far, the dominant species of dirofilariosis in the country.

In Chapter 2, scientific publication 3, a sensitive species-specific PCR assay targeting filarial genomic DNA was optimized and used for the simultaneous detection and differentiation of *D. immitis*, *D. repens* and other concurrent filarial species in animal reservoirs. Two different PCR protocols were used to amplify ribosomal Internal Transcribed Spacer (ITS) regions 1 and 2, showing a higher analytical sensitivity for ITS2. Although parasitological and serological methods are still the most common techniques used for the diagnosis of canine dirofilariosis, results showed that ITS2-PCR performs better due to its increased sensitivity, specificity and ability to identify species, representing a valuable tool for the diagnosis and screening of filarial infections in dogs.

In Chapter 2, scientific publication 4, the first nationwide serological survey was performed to assess *A. vasorum* infections in dogs in mainland Portugal. New, highly sensitive and specific ELISAs were used to detect *A. vasorum* circulating antigen and specific antibodies against the parasite. Results showed a total of 0.7% positives simultaneously for *A. vasorum* circulating antigen and specific antibodies. These animals were detected in northern, central and southern areas of Portugal, indicating active infections widely distributed throughout the country. Additionally, 1.3% were *A. vasorum* antibody-positive, suggesting previous exposure to the parasite. The endemic occurrence of *A. vasorum* in dogs from different geographical areas of Portugal was therefore confirmed.

Overall, the seroprevalence of CPA in Portugal (0.7%, regarded as the positivity for both *A. vasorum* circulating antigen and specific antibodies against the parasite) is similar to that reported in other European countries, such as Germany (0.3%) (Schnyder et al., 2013a), Sweden (0.4%) (Grandi et al., 2016), Poland (0.5%) (Schnyder et al., 2013b), Italy (0.8-0.9%) (Guardone et al., 2013), Switzerland (0.96%) (Lurati et al., 2015), UK (0.97%) (Schnyder et al., 2013a) and Hungary (1.4%) (Schnyder et al., 2015a).

This first nationwide survey for *A. vasorum* was performed in dogs using new sensitive ELISAs, confirming its endemic occurrence in dogs from different geographical areas of Portugal, with an overall prevalence of 0.7%.

Military working dogs constitute a risk group for CVBDs due to their frequent outdoor work in different areas and consequent increased exposition to arthropod vectors. To ascertain their exposure, a survey was conducted involving Portuguese Air Force dogs (Chapter 2, scientific publication 5). They were serologically tested for *D. immitis* and *A. vasorum,* as well as seven other vector-borne agents. Although no positives were recorded for *D. immitis* antigen, specific antibodies against *A. vasorum* were detected in 5% of the military dogs, including one case simultaneously positive for *A. vasorum* circulating antigens, denoting an ongoing infection. This study showed a southward trend of increased pathogen diversity and brought new information concerning the presence of *A. vasorum* and its geographical distribution in Portugal.

Dogs infected with *D. immitis* and *A. vasorum* were found in northern, central and southern regions of Portugal, although with a higher prevalence of *D. immitis*. Though exposure may differ depending on the geographical region of Portugal, the likelihood of dirofilariosis and angiostrongylosis is considerable nationwide. Considering the ongoing changes in climate, transport and demography, dog exposure to infection seems likely to increase in the future, in either endemic and non-endemic areas.

In Chapter 3, scientific publication 1, the author aimed to characterize the current prevalence and distribution of *D. immitis* and *A. vasorum* in foxes (*Vulpes vulpes)*, considered as powerful sylvatic reservoirs and spreaders of both parasites. Vulpine populations from eight districts of Portugal were tested by serological analysis. Overall, 12.7% foxes were positive for *A. vasorum*, with 5.9% positive for both *A. vasorum* antigen and antibody detection, 5.1% only *A. vasorum* antigen-positive and 1.7% only *A. vasorum* antibody-positive. In addition, 8.5% were positive to the *D. immitis* antigen test. Positive animals for CPA and CPD were detected in northern and southern areas of Portugal.

The prevalence registered is in line with the one reported in previous studies conducted by necropsy, where *D. immitis* prevalence was found to vary in Portugal between 3.2% and 11.8% and *A. vasorum* prevalence between 0.33% to 16.1% (Carvalho-Varela & Marcos, 1993; Eira et al., 2006).

A survey conducted in vulpine populations from eight districts of Portugal found 12.7% positive for *A. vasorum* and 8.5% positive for *D. immitis*, tested by serological analysis.

In Chapter 3, scientific publication 2, infection by *D. immitis* was reported in three pinniped species: common seals (*Phoca vitulina*), California sea lions (*Zalophus californianus*) and South African fur seals (*Arctocephalus pusillus pusillus*) kept at an oceanographic park in the Algarve. Before this study, *D. immitis* had only been found in hooded seals (*Cystophora cristata*), common seals (*Phoca vitulina*) and California sea lions (*Zalophus californianus*). This discovery resulted in a publication reporting the South African fur seal (*Arctocephalus pusillus pusillus*) as a new host for *D. immitis* infection. Additionally, 43.8% of the pinnipeds surveyed were positive for *D. immitis* by real-time PCR, 12.5% positive for the nematode's antigen and 6.3% had *D. immitis* microfilariae. This high prevalence detected in a confined area located in a popular summer destination may represent a risk interface for zoonotic pathogen transmission, and an example of how a One Health approach is vital to improve early diagnosis and control of zoonotic pathogens in humans and wildlife. As no epidemiological studies on pinniped populations were available and only few cases of infection have been described thus far, this study brought new information on the occurrence, distribution and diagnosis of *D. immitis* in these carnivores.

A high prevalence of *D. immitis* was detected in a pinniped collection at an oceanographic tourist park in the Algarve. The South African fur seal was reported as a new host for *D. immitis*.

In Chapter 4, DNA of the endosymbiont bacterium *W. pipientis* was detected for the first time in Portuguese canine populations naturally infected with *D. immitis*. Overall, *Wolbachia* DNA was detected by PCR in 52.6% of the canine blood samples tested, a prevalence higher than the 30.6% reported in Spain (Tabar, Altet, Martínez & Roura, 2013), but lower than the 100% found in Brazil (Rossi et al., 2010) and the 64.0% recently reported in dogs in southern Portugal (Maia et al., 2016b). Considering the pivotal implications of *Wolbachia* organisms on the pathogenesis of filarial diseases, antibiotic therapy should be used as adjunct therapy for canine dirofilariosis to reduce the overall pathological side effects.

DNA of *Wolbachia pipientis*, the intracellular endosymbiont bacterium of filarial nematodes, was detected for the first time in dogs naturally infected with *D. immitis* in Portugal.

In chapter 5, *Dirofilaria* transmission risk was assessed using a degree-day model, based on the temperatures registered at five meteorological stations in Portugal over a decade. Overall, Madeira Island was found to be the area registering the highest number of days with suitable conditions for *D. immitis* transmission (an average of 209.9 days per year), followed by Faro (175.2 days/year), Lisbon (163.5 days/year), Azores (140 days/year) and Oporto (117.2 days/year). The average risk period was 8 months/year in Madeira, 6.9 in Faro, 6.4 in Lisbon, 5.6 in Azores and 5 in Oporto. As expected, higher average temperatures were observed predominantly in Madeira and in the southern parts of the country. These higher temperatures raise the transmission risk of *Dirofilaria*, as they enable a faster extrinsic development of larvae in the mosquitoes and a higher number of parasite transmission cycles. Although risk was found to be markedly seasonal, predominantly in the summer, this study evidenced that in Portugal the risk period is starting earlier and lasting far beyond the warmest months of the year. Overall, the filarial transmission season was found to be longer than the ones previously described by Genchi et al., (2005). This is in line with the "seasonality paradigm" of vector-borne diseases, which shows that their occurrence is no longer a seasonal phenomenon (Otranto et al., 2013). Thus, effective protection against vectors over an extended period is needed, particularly in regions where the temperature is frequently above 14°C. Considering the high prevalence of infected dogs across the country, the presence of competent mosquito vectors, and Portugal's warm climate throughout the year, a continued year-round prophylactic approach is strongly recommended. It's also important to highlight that the involvement of vector mosquitoes in the life cycle of *Dirofilaria* spp. makes their transmission and distribution susceptible to climate changes, as well as to rapid and significant variations in defined geographic regions. Presently, we are facing an increasing risk of vector-borne pathogens in the country as predicted by Casimiro, Calheiros, Santos and Kovats (2006), which may become worse with the introduction and spread of vectors such as *Aedes albopictus, Aedes japonicus* and *Aedes koreicus* (Montarsi et al., 2015; Silaghi et al., 2017).

Although the transmission of *D. immitis* in Portugal is markedly seasonal, with a peak in the summer, the risk period is starting earlier and lasting far beyond the warmest months of the year.

As previously referred, canine dirofilariosis is a severe and life-threatening parasitic disease. Its main treatment goals are to improve the animal's clinical condition and to eliminate all life stages of the parasite with minimal post treatment side effects. This can be achieved through mechanical, surgical or chemotherapeutic approaches. Chemotherapy (based on melarsomine

dihydrochloride, MLs and doxycycline) can lead to several complications and adverse effects, including pulmonary thromboembolism and anaphylactic shock (Atkins, 2010). For this reason, either mechanical or surgical heartworm removal methods are generally preferred to eliminate as many adult worms as possible before pharmacological treatment is initiated. Manual extraction is the preferred method due to its diminished invasiveness, reduced damage to the vascular endothelium and shortened anaesthesia duration (Atkins, 2010; Bové et al., 2010). However, it remains an expensive technique that can be highly traumatic. To overcome this issue, in Chapter 6, a new minimally invasive surgical technique for mechanical removal of *D. immitis* was developed using a non-traumatic catheter-guided snare. A 0.014-inch coronary wire (BMW, Abbott Vascular) was adapted, allowing the successful transvenous extraction of *D. immitis* adult specimens from the pulmonary artery and right ventricle of a severely infected dog. Further surgical interventions need to be done to improve the efficiency of this technique. Nevertheless, we believe that the possible cost reductions and reduced traumatic damage induced by this snare (when compared to existing alternatives), will allow heartworm extraction to be more affordable and consequently widespread, thereby promoting the treatment of a larger number of animals, enhancing a specific chemotherapy with higher safety.

A new minimally invasive surgical technique was developed, creating an alternative method for mechanical extraction of *D. immitis* adult worms from the hearts and pulmonary arteries of dogs, through transjugular catheterization, using a non-traumatic catheter-guided snare.

In Chapter 7, the authors assessed parasite control practices and public perceptions of pet owners through a questionnaire conducted at the Small Animal Hospital (Faculdade de Medicina Veterinária, Universidade de Lisboa). The authors found that although most Portuguese pet owners give antiparasitic drugs to their dogs, the majority deworm them at irregular and consequently ineffective intervals. Despite the availability of a variety of antiparasitic prophylactic measures, only 11.8% of the dogs were under the recommended endoparasitic treatment (i.e. quarterly, at least) and only 28.4% were continuously protected throughout the year from vector-borne agents. Additionally, 60% of the owners that kept their dogs outdoors all day, did not provide adequate ectoparasitic prevention for their animals.

It is important to highlight that the prophylactic use of MLs-based products is vital to control *D. immitis* in dogs. Monthly administration of topical or oral MLs (or of the 6 months slow-release formulation) has shown to be effective for prevention and is recommended. Control measures to prevent mosquito bites are also crucial to reduce *D. immitis* infection, namely:

regular application of mosquito repellents, emptying standing water collections, installation of window screens and avoidance of areas and periods of the day when mosquitoes are most active (ESCCAP, 2012). To control *A. vasorum*, strategies indicated to reduce the risk of infection with L3 larvae include: preventing dogs from ingesting snails or slugs, avoid leaving dogs outside the house or feeding them outside, and regularly cleaning dogs' bowls and toys to make them less accessible to slugs and snails (Elsheikha et al., 2014).

Although most Portuguese owners give antiparasitic drugs to their dogs, the majority deworm them at irregular and consequently ineffective intervals, with only 11.8% under the recommended endoparasitic treatment and only 28.4% continuously protected throughout the year from vector-borne agents.

Furthermore, the preventative safety measures taken by dog owners in public places are still insufficient with 37% answering they did not collect their dog faeces in all public places. This may potentially contribute to environmental contamination with *A. vasorum* L1, among others parasitic agents. The necessity to implement environmental control measures (namely dog faeces removal from public places) along with effective worm control is fundamental, to minimize environmental contamination and safeguard public and animal health (ESCCAP, 2012).

The level of public knowledge about parasites and parasitic diseases is still low in the country, i.e., 88% had never heard of "dirofilariosis/heartworm disease" and 85% had never heard of the word "zoonosis". One third of the owners were unable to cite any possible source of parasitic infection. In another survey conducted in Portugal, although 56.5% of the owners had heard of the term "zoonosis/zoonoses", only 35.2% knew its real meaning and 50.8% were unaware of "dirofilariosis/heartworm disease" (Pereira et al., 2016). The general lack of owners' knowledge regarding parasitic infections and adequate control schemes highlights the key role that veterinarians have in providing information to owners and in raising public awareness. The implementation of continued ectoparasiticide prophylaxis is crucial, especially in high-risk CVBD areas, like southern European countries, including Portugal. It is also relevant to perform screenings for *D. immitis* and *A. vasorum* infections on a regular basis, especially given the chronic progression and subclinical character of the induced diseases (McCall et al., 2008b; Koch & Willesen, 2009). For *D. immitis*, dogs should be checked for both circulating antigens and blood microfilariae at the beginning of each preventative annual treatment (ESCCAP, 2012). For *A. vasorum*, dogs should be checked for the presence of L1 in faeces collected over three consecutive days using the Baermann's test (ESCCAP, 2012), or using the commercial

test for the detection of *A. vasorum* circulating antigens in clinically suspected animals (Schnyder et al., 2014).

In chapter 8, a full revision of the epidemiological situation of CPD and CPA in canids in Portugal was performed, providing a comprehensive update of the past 20 years.

9.2 Limitations of the study and future perspectives

Despite the broad epidemiological survey here conducted, due to financial constraints, it was not possible to assess all the geographical areas in Portugal. It would have been important to perform a full characterization of the situation in the entire country, covering all mainland districts, as well as the autonomous regions of the Azores and Madeira. In addition, it would have been relevant to extend the study to pet dogs and other risk groups, such as hunting and shepherd canids that may also be relevant reservoirs of infection. Similarly, it would have been interesting to extend the survey to other carnivores such as the endangered Iberian wolf, allowing an even better understanding of major heartworms in domestic and wild canids. Despite the author's continued efforts to contact local and national authorities and conservation groups, this was not possible as no samples were available.

Considering the potential serological cross reactions between *D. immitis* and *A. vasorum*, it would have been important to test canids simultaneously for both agents. Unfortunately, for logistical reasons and a lack of financial support, this was not possible. Moreover, considering that felids may also be affected with dirofilariosis, and given the scant information available regarding the situation of feline cardiopulmonary and subcutaneous dirofilariosis, a large-scale study would have also been pertinent.

Although temperatures are one factor that influence the transmission of *Dirofilaria* spp., many other factors may interfere with its spread, such as precipitation, relative humidity, vegetation indexes, human and animal population density, and social economic status. Furthermore, model-based predictions fail to consider the influence of microclimates, variations in larval development times, adaptations of the mosquito vectors, mosquito life expectancy ranges and temperature fluctuations. Climate models that gather all these factors should be developed and combined with the prevalence data collected on epidemiological studies over the years, to give a more accurate perspective of the ongoing situation.

Despite the high prevalence and wide distribution of canid dirofilariosis, as well as the presence of vectors in Portugal, information regarding human dirofilariasis is almost inexistent, with only sporadic cases being documented. This is quite intriguing when considering the increasing number of human infections by *D. immitis* and *D. repens* reported in neighbouring countries.

Epidemiological studies with specific anti-*Dirofilaria* antibodies should be carried out in human populations, particularly on those living in endemic areas, to ascertain the rates of seropositivity. This would allow researchers to detect previous contact with the parasite and thus, to clarify the impact of *Dirofilaria* on humans in Portugal. Similarly, a stronger inter-sectoral collaboration between public health and veterinary institutions should be fostered and awareness campaigns performed to increase the knowledge on *Dirofilaria* amongst medical professionals.

Regarding *A. vasorum*, there are currently no reports concerning its intermediate host in Portugal. Further studies are needed to clarify the role and importance of the diversified spectrum of intermediate hosts, but also its paratenic hosts. Considering that coagulopathies are one of the most important factors leading to fatalities in dogs infected with *A. vasorum*, research on the mechanisms leading to this disorder and the parasite molecules involved in coagulopathy is crucial.

A greater understanding of these issues will enable earlier diagnosis, better surveillance and the development of more tailored control and treatment measures against major canid heartworms.

9.3 Conclusions

All the major goals proposed for this thesis were successfully accomplished, addressing the lack of knowledge about fundamental aspects regarding the epidemiological distribution of major heartworms in carnivores in Portugal.

Important contributions to the knowledge of dirofilariosis and angiostrongylosis in Portugal were achieved. The high prevalence detected coupled with the forecasted trends and the lack of pet owners' knowledge, highlight a crucial necessity to raise public awareness and overcome rooted misconceptions. Our data will be critical to monitor and forecast future heartworm epidemiological trends, ensuring a continued surveillance and an integrated One Health approach. Considering the ongoing changes in climate and ecosystems, dog exposure to infection seems likely to increase in the future, in either endemic and non-endemic areas. Therefore, it is crucial to alert veterinarians to take cardiopulmonary nematodes into consideration in their diagnostic routine, and to promote effective preventive and control measures to improve the diagnosis and the spread of both diseases.

CHAPTER 10

References

References*

*(The bibliographic references presented in chapter 10 refer to chapters 1 and 9. The bibliographic references presented in each scientific publication are referred at the end of each publication to facilitate readers).

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